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PRINCIPAL INVESTIGATOR: Linda M. Varela
Margot M. Ip, Ph.D.

CONTRACTING ORGANIZATION: Health Research, Inc.
Buffalo, New York 14263

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Margot M. Ip, Ph.D.

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The pleiotropic cytokine tumor necrosis factor-alpha (TNF) has previously been shown to regulate both the proliferation and differentiation of normal rat mammary epithelial cells (MEC) in primary culture. TNF and TNF receptor expression were measured in isolated MEC and found to be independently and specifically regulated during mammary gland development. Using agonistic antibodies to either TNF receptor, the individual roles of each receptor were investigated in MEC in primary culture. The p55 TNF receptor was found to be the sole mediator of TNF-induced proliferation, while the two receptors were found to have opposing effects on functional differentiation. Further studies were undertaken to determine whether the post-receptor pathway of TNF action in MEC involved subsequent signaling via the EGF receptor. Using an inhibitor of the EGF receptor tyrosine kinase activity, PD158780, it was determined that the EGF receptor is not necessary for TNF action in normal MEC. Lastly, TNF and TNF receptor expression were measured in both DMBA- and NMU-induced mammary tumors, and the effects of TNF on the growth and morphogenesis and NMU-initiated and -transformed MEC in culture were determined.

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FOREWORD

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Linda M. Varela 6/24/98
PI - Signature Date

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Address to Statement of Work:

All aspects of the Statement of Work were addressed as follows:

Task 1. *Investigation of TNFα production by mammary epithelial cells.* It was determined that normal MEC produce TNFα, and changes in TNFα mRNA and protein expression were assessed during the various stages of normal *in vivo* mammary gland development (puberty, pregnancy, lactation and involution). Due to technical difficulties, it was not possible to examine the hormonal regulation of TNFα production by MEC *in vitro*.

Task 2. *Studies of TNF receptors.* It was determined that MEC express mRNA transcripts for both the p55 and p75 TNF receptors, and the pattern of expression of these receptor transcripts during the various stages of *in vivo* mammary gland development was identified; it was not possible to determine the profile of receptor protein expression. The functions of the individual TNF receptors in directing normal MEC growth and development were also identified using specific agonistic antibodies directed against each of the receptors.

Task 3. *Comparison with transformed mammary epithelial cells.* Alterations in TNFα and TNF receptor expression in DMBA- and NMU-transformed MEC (compared to normal MEC) were identified. The effects of TNFα on the growth and morphological development of NMU-initiated and -transformed MEC were examined.

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Publications:

L.M. Varela, and M.M. Ip. (1996) Tumor necrosis factor α: A multifunctional regulator of mammary gland development. *Endocrinology* **137**: 4915-4924.

L.M. Varela, K.M. Darcy and M.M. Ip. (1997) The epidermal growth factor receptor is not required for TNFα action in normal mammary epithelial cells. *Endocrinology* **138:** 3891-3900.

Manuscripts Submitted:

K.M. Darcy, A.L. Wohlheuter, D. Zangani, P.A. Masso-Welch, M.M. Vaughn, J.A. Russell, L.M. Varela, S.M. Shoemaker, E. Horn, P.-P.H. Lee, R.-Y. Huang, and M.M. Ip. Selective changes in EGF receptor expression and function during the proliferation, differentiation and apoptosis of mammary epithelial cells.

Abstracts:

L.M. Varela, and M.M. Ip. The p55 and p75 TNF receptors both signal functional responses in normal rat mammary epithelial cells. Published in the *Proceedings of the Annual Meeting of the American Association of Cancer Research* **36**: A235, 1995.

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L.M. Varela, and M.M. Ip. The role of TNFα in normal mammary gland development. Presented at the Gordon Research Conference on Mammary Gland Biology. New London, NH, June 1995.

E. Mihich, M.J. Ehrke, A. Henn, E. Berleth, L. Varela, and M.M. Ip. Therapeutic potential of tumor necrosis factor (TNF): Basic studies. Presented at a European School of Oncology Symposium. Cairo, Egypt, January 1996.

M.M. Ip, L. Varela, S. Shoemaker, W. Shea, and K. Darcy. Regulatory role of tumor necrosis factor-α in mammary gland development. Presented at the International Association for Breast Cancer Research meeting. Paris, France, July 1996.

L.M. Varela, K.M. Darcy and M.M. Ip. The epidermal growth factor receptor (EGFR) does not play a significant role in the pathway of TNFα action in normal mammary epithelial cells (MEC). Published in the *Proceedings of the Annual Meeting of the American Association of Cancer Research* **38**: A379, 1997.

K.M. Darcy, A. Wohlheuter, S. Shoemaker, P.-P. Lee, L. Varela, and M.M. Ip. Differential effects of epidermal growth factor receptor (EGFR) inhibition in mammary epithelial cells. Published in the *Proceedings of the Annual Meeting of the American Association of Cancer Research* **38**: A376, 1997.

INTRODUCTION

The risk that a woman in the U.S. will develop breast cancer in her lifetime has now increased to an unprecedented one out of eight women, and breast cancer is currently the most common cancer among women. It is therefore crucial that a better understanding of those factors leading to the development of breast cancer and of the metastatic phenotype be achieved so that more appropriate strategies for its prevention and/or treatment can be applied. This can be achieved by a thorough investigation of the normal mammary gland, and through a subsequent comparison of this with malignant breast cells, important clues as to how breast cancer evolves may be discovered. While the fundamental causes for breast cancer remain elusive, a growing body of data suggests that the major risk factors may be inherently biological, such as natural hormone or growth factor production (1). Thus, by examining the cyclical variations in hormone levels, the complex hormonal regulation of proliferation and differentiation, and the changes in gene expression that occur as the mammary gland progresses through the different stages of development, the prospects for prediction, prevention, and treatment of breast cancer may be heightened. A possible key player in this intricate network is tumor necrosis factor - alpha (TNFα). TNFα has been shown by our laboratory to play a significant role in directing both the proliferation as well as the morphological and functional differentiation of mammary epithelial cells (2). This regulator may reach the MEC not only via traditional endocrine and local paracrine routes, but there may also be autocrine synthesis of TNFα by the MEC as well. Normally, there is strict control of the expression of this cytokine; however, it is possible that any disruption of this control has the potential to markedly affect the degree of both growth and differentiation and may confer on the cell a transformed phenotype. Thus, it is critically important to determine the physiological role of TNFα in the growth and development of the mammary gland and how this role differs in transformed cells, so that more appropriate strategies for the prediction, prevention, and treatment of breast cancer may be developed.

Background: TNFα is a multifunctional cytokine that was originally defined by its ability to cause the hemorrhagic necrosis of tumors *in vivo*. It is now known, however, that TNFα affects the growth, differentiation, and/or function of virtually all cell types, either by acting alone or in concert with a variety of other cytokines, hormones or growth factors (3-6). On the first level, this complex physiology may be the result of different forms of TNFα, each of which has significant activity (7,8). Specifically, TNFα is first synthesized as a 26-kDa transmembrane precursor which is then proteolytically cleaved to release the 17-kDa soluble cytokine. In addition, TNFα production is under strict regulation; expression is controlled by numerous variables, including hormones and cytokines, and there is also stringent post-transcriptional regulation (9-11). Lastly, the pleiotropic effects of TNFα may be mediated through different receptors. Two distinct cell surface TNF receptors of 55- and 75-kDa (p55 and p75, respectively) have been identified in varying proportions on the membranes of virtually all cells so far examined (12-14). Although these two receptor forms share sequence homology in their extracellular domains, they have no significant homology in their intracellular regions and have unique postreceptor signaling pathways which primarily induce distinct responses. For example, a large majority of TNFα activities, including cytotoxicity (15,16), antiviral activity (17), stimulation of fibroblast proliferation (18), and up-regulation of epidermal growth factor receptor mRNA in epithelial cells (19) are mediated by p55, while the 75-kDa TNF receptor transduces signals for thymocyte proliferation (15), inhibition of hematopoiesis (20) and up-regulation of both transforming growth factor α (TGFα) and granulocyte macrophage-colony stimulating factor (GM-CSF) secretion (19,21). In some systems, however, the two receptors

are also capable of inducing the same effects. Agonistic antibodies directed against either TNF receptor were able to induce apoptosis in human rhabdomyosarcoma cells (22), and both TNF receptors share the ability to activate the transcription factor NFκB; however, the time course of NFκB activation is different for each receptor, so it is believed that the signaling pathways of p55 and p75 may be distinct (23).

Previous studies demonstrated the presence of TNF receptors on both normal and malignant mammary cells (24) and determined that human breast cancer cells in culture were sensitive to either the cytostatic or cytotoxic effects of TNFα (5,24,25). In contrast, earlier work by our laboratory demonstrated that TNFα actually *stimulates* the growth and morphological development and regulates the function of normal mammary epithelial cells (MEC) in culture (2). These studies used a primary culture model system developed by our laboratory in which undifferentiated MEC, cultured in the presence of a defined, serum-free medium, proliferate, morphologically develop, and functionally differentiate to an extent comparable to that of cells within the mammary gland of a rat during lactation (26-28). Specifically, TNFα was found to stimulate MEC proliferation in both the presence and absence of epidermal growth factor (EGF), a major growth and differentiation factor for the MEC in this culture system. Both TNFα and EGF also stimulate the morphological development of the MEC organoids, inducing the formation of large, well-differentiated alveolar colonies which, in the case of TNFα, had extensive ductal branching (2). Lastly, TNFα modulated the functional differentiation (casein accumulation) of the MEC organoids in culture; however, these effects were more complex than its effects on either growth or morphogenesis. Higher concentration of TNFα inhibited casein accumulation, but in the absence of EGF, lower concentrations of TNF were found to stimulate casein accumulation at later times in culture. Thus, these experiments suggested that TNFα may play a physiological role in normal mammary gland development.

If TNFα is indeed a biological regulator of normal mammary gland development, it would be expected that either the MEC or the stromal cells of the mammary gland would produce this cytokine, and that expression of both TNFα and its receptors would be developmentally (and thus hormonally) regulated. Therefore, the expression of TNFα and its two receptors was examined in freshly isolated rat MEC during puberty, pregnancy, lactation, and post-lactational mammary gland involution, and attempts were made to determine the level of biologically active TNFα during these various stages. In addition, the functional roles of both the p55 and p75 TNF receptors in normal MEC growth and differentiation were investigated using agonistic antibodies specific for each receptor. Furthermore, one potential postreceptor pathway of TNFα action in normal MEC was investigated. Given the documented interaction between TNFα and the EGF receptor in other cell types, and because of the similarity of some of the actions of TNFα and EGF in normal MEC, we postulated that the EGF receptor may mediate the effects of TNFα on growth and possibly on differentiation of normal MEC. Finally, TNFα and TNF receptor expression may also be altered in mammary tumors; therefore, expression was examined in DMBA- and NMU-induced mammary tumors and compared to expression in normal MEC, and the effects of TNFα on the growth and differentiation of NMU-induced mammary tumor cells in culture were examined.

MATERIALS AND METHODS

Materials

Dispase grade II powder (neutral protease), leupeptin, glycine and guanidine hydrochloride were purchased from Boehringer-Mannheim (Indianapolis, IN), collagenase class III was obtained from Worthington Biochemical Corp. (Freehold, NJ), liquid dispase (50 caseinolytic units/ml) was obtained from Collaborative Biomedical Products (Bedford, MA), MOPS [3-(N-Morpholino)propanesulfonic acid, sodium] was purchased from Calbiochem (Cambridge, MA), and newborn calf serum (NCS), gentamicin, phenol red-free RPMI-1640, and Trizol were purchased from Life Technologies (Grand Island, NY). Fetal bovine serum (FBS) was a product of Hyclone Laboratories, Inc. (Logan, UT), HEPES was from United States Biochemical (Cleveland, OH), and SDS was purchased from Biorad (Hercules, CA). Diaminobenzidine (DAB), fatty acid-free bovine serum albumin (BSA), phenol red-free Dulbecco's Modified Eagle's Medium-F12 (DMEM-F12, 1:1) containing 15 mM HEPES, methionine-free DMEM-F12 (1:1, containing 15 mM HEPES), insulin, progesterone, hydrocortisone, ascorbic acid, apo-transferrin, NP-40, 3-(4,5-dimethylthiazol-2-yl)-2,5-di-phenyltetrazolium bromide (MTT), dithiothreitol (DTT), 2,5-diphenyl-oxazole (PPO), urea, agarose, trichloroacetic acid (TCA), sodium phosphate, aprotinin, pepstatin A, benzamidine, iodoacetamide, sodium deoxycholate, sodium azide, glutamine, N-ethyl-maleimide (NEM) and diethyl pyrocarbonate (DEPC) were purchased from Sigma Chemical Co. (St. Louis, MO). Ovine prolactin (NIDDK-oPL-20) was a gift from the NIDDK-NIH (Bethesda, MD) and mouse epidermal growth factor (EGF) was purchased from Upstate Biotechnology (Lake Placid, NY). Goat anti-rabbit biotinylated immunoglobulin G (IgG) and streptavidin-peroxidase were products of Chemicon International, Inc. (El Segundo, CA), and chloroform, formaldehyde, methanol, DMSO and isopropanol were obtained from Fisher Scientific (Fair Lawn, NJ). Donkey anti-rabbit peroxidase-conjugated IgG was a product of Jackson ImmunoResearch Labs. (West Grove, PA). Peroxidase conjugated goat anti-hamster IgG and biotinylated goat anti-hamster IgG were procured from Caltag (So. San Francisco, CA), and streptavidin-peroxidase (for the TNF receptor Western blot analysis) from Zymed (So. San Francisco, CA). [Methyl-^{3}H]-thymidine, [^{125}I]-Bolton-Hunter labeled TNFα and [α-^{32}P]-deoxycytidine 5'-triphosphate were purchased from DuPont New England Nuclear (Boston, MA). Tran ^{35}S-label containing ^{35}S-L-methionine and ^{35}S-L-cysteine was a product of ICN Biomedicals, Inc. (Costa Mesa, CA), and protein A Sepharose 6MB was from Pharmacia Biotech (Uppsala, Sweden). The Multiprime labeling kit used to radiolabel the complementary DNA (cDNA) probes was purchased from Amersham (Arlington Heights, IL). The cDNA probe for recombinant human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was purchased from Clontech (Palo Alto, CA). The J774A.1 mouse macrophage cells were purchased from ATCC (Rockville, MD). Recombinant human TNFα (2.5 x 10^{6} U/mg), recombinant murine TNFα (3.1 x 10^{6} U/mg) and plasmid pRtac1 containing a 1 kilobase (kb) rat TNFα cDNA insert (29) were generous gifts of Asahi Chemical Industry Co. (Fuji, Shizuoka, Japan). Plasmids containing the 1.8 kb and ~1.0 kb cDNA inserts corresponding to the murine p55 and p75 TNF receptors, respectively (30), were gifts of Genentech (San Francisco, CA), as were the two agonistic antibodies specific for either the p55 or p75 TNF receptor (15). The cDNA probe for the rat cyclophilin gene (p1B15) (31) was generously provided by Dr. Henry Thompson (AMC Cancer Research Center, Denver, CO). The monoclonal hamster anti-mouse p55 TNF receptor antibodies were a generous gift of Dr. Robert Schreiber (Washington University, St. Louis, MO), and the anti-human p55 TNF receptor antibody was kindly provided by Dr. M. Brockhaus (Hoffmann-LaRoche, Basel,

Switzerland). The inhibitor of the EGF receptor tyrosine kinase activity, PD 158780, was generously provided by Dr. David Fry at Parke-Davis (Ann Arbor, MI); this compound has poor inhibitory activity against PDGF or FGF receptor tyrosine kinases but is a potent inhibitor of the tyrosine kinase activity of the EGF receptor and the other EGF receptor family members, erbB2, B3 and B4. Wehi 164 mouse fibrosarcoma cells, phytohemagglutinin, recombinant murine IL-1β, and both rat and human IL-2 were gifts of M. Jane Ehrke and Dr. Enrico Mihich.

Animals

For the primary culture of MEC, virgin, 50- to 55-day-old female Sprague-Dawley Crl:CD BR rats were purchased from Charles River (Kingston, NY) and used as the source of mammary glands. For isolation of MEC for analysis of TNFα and TNF receptor mRNAs, pregnant Sprague-Dawley Crl:CD BR rats were purchased from Charles River, and either sacrificed during mid-pregnancy (days 13-14) or allowed to give birth. Rats were then sacrificed on day 5, 10 or 15 of lactation, or either 12 hours or 1 week after the pups had been removed on day 21 of lactation. Virgin rats, 50-55 days of age, were used as controls. Six month old virgin female rats were used as the source of control mammary glands for the study of TNFα and TNF receptor expression in DMBA- and NMU-induced mammary carcinomas. Female CD2F1 mice, purchased from NCI-Frederick Cancer Research Facility, Biological Testing Branch (Frederick, MD), were used to passage the Engelbreth-Holm-Swarm (EHS) sarcoma, which was the source of the reconstituted basement membrane (RBM) matrix for the primary culture of the MEC organoids. Armenian hamsters, from which the normal hamster serum for the TNF receptor Western blot analysis was obtained, were purchased from Cytogen Research and Development (West Roxbury, MA). Virgin rats and mice were fed 6% fat chow diets, while rats during pregnancy and lactation were fed chow diets containing 10% fat (Teklad, Madison, WI). All animals were fed *ad libitum* and had free access to water. Animal rooms were air conditioned and humidity controlled, with light cycles of 14 h on-10 h off (rats) or 12 h on-12 h off (mice). The care and use of the animals was in accordance with NIH guidelines and Institute Animal Care and Use Committee regulations.

Preparation of the reconstituted basement membrane (RBM) matrix

The RBM matrix used for the primary culture of the MEC organoids was extracted from the Engelbreth-Holm-Swarm (EHS) sarcoma as previously described (26,32).

Mammary epithelial cell organoid isolation

For analysis of TNFα and TNF receptors in freshly isolated MEC, mammary glands were excised from virgin, mid-pregnant (days 13-14), lactating (days 5, 10, and 15) or post-lactational (either 12 hours or day 7 of involution) female rats, mechanically minced and placed in a digestion solution (10 ml/g wet wt) consisting of 0.2% (w/v) collagenase type III, 0.2% (w/v) dispase II, 5% (v/v) NCS, and 50 μg/ml gentamicin in phenol red-free RPMI 1640 (0.45μm filter-sterilized). The mammary gland suspensions were then incubated at 37°C in 5% CO_2, 95% air for approximately 13.5 hours for virgin glands or 3-4 hours for pregnant, lactating and post-lactational glands. The digestion was judged to be complete when the tissue had been digested to small clusters of epithelial cells which are denoted as MEC organoids. The resultant epithelial cell organoids were pelleted by centrifugation at 500 x *g* for 10 minutes at 25°C, washed once with DMEM-F12, re-centrifuged at 500 x *g* for 10 minutes, and each pellet was resuspended in 45 mls of DMEM-F12. The MEC suspension was then filtered through a 530 μm nitex filter (Tetko, Depew, NY) to remove any large aggregates of glandular material

and was then passed through a 60 μm filter. The 60 μm filter trapped the epithelial organoids but allowed the passage (and removal) of small cell clusters and single cells. The MEC organoids isolated from pregnant, lactating or post-lactational animals were rinsed off the 60 μm filter with a 0.45 μm filter-sterilized solution of 10% (v/v) NCS and 50 μg/ ml gentamicin in phenol red-free DMEM-F12, while organoids isolated from the mammary glands of virgin animals were rinsed off the filter with phenol red-free DMEM-F12 containing 5% (v/v) NCS and 50 μg/ml gentamicin. All organoid suspensions were then refiltered through the 530 μm mesh and incubated in a plastic tissue culture flask for 2.5 hours (for MEC from pregnant, lactating or post-lactational animals) or 4 hours (MEC from virgin animals) at 37°C in 5% CO_2, 95% air to facilitate that attachment and subsequent removal of any remaining stromal cells from the non-adherent MEC organoids. The number of MEC within the organoids was then enumerated by counting of nuclei as previously described (32).

RNA isolation and Northern blot analysis

The freshly isolated epithelial organoids from all developmental stages were pelleted by centrifugation at 500 x *g* for 10 minutes. The cell pellet was resuspended in Trizol, a phenol-based reagent capable of extracting both RNA and protein from the same cells, at a concentration of 10^7 cells per ml Trizol, and the MEC organoids were sheared through an 18 gauge needle. Total cellular RNA and protein (see below) were then prepared following the protocol of the manufacturer (Life Technologies), and polyadenylated (poly A^+) mRNA was subsequently fractionated using the PolyATract mRNA Isolation System (Promega, Madison, WI). Approximately 1.0 - 1.5 μg of denatured poly A^+ mRNA (dissolved in ribonuclease-free water) was separated by electrophoresis on a 1% (w/v) agarose gel containing 1 X MOPS and 2.2 M formaldehyde overnight at 37 volts (room temperature). To determine the size of mRNA transcripts, a 0.24 - 9.5 kb RNA ladder (Life Technologies) was also included on the gel. Before electrophoresis, gels were pre-electrophoresed for 10 minutes at 35 volts. The electrophoresis running buffer, which consisted of 0.72 M MOPS, 0.01 M Na acetate (pH 5.2), and 1 mM EDTA (pH 8.0) prepared in DEPC-treated water to a final pH of 7.0, was circulated during electrophoresis with a Polystaltic pump (Buchler). After electrophoresis, the gels were soaked in three changes of DEPC-treated water (10 minutes each) to remove excess formaldehyde, and the RNA was then transferred to a Hybond N nylon membrane (Amersham) following standard procedures. The RNA was cross-linked to the membranes by UV irradiation (UV Stratalinker 1800, Stratagene, La Jolla, CA). Northern blots were prehybridized for 2 hrs at 65°C in a buffer of 0.5 M Na phosphate, pH 7.2, 7% (w/v) SDS, 1% (w/v) BSA, and 1 mM EDTA according to the method of Church and Gilbert (33). The cDNA probes for TNFα and the p55- and p75-TNF receptors were random-prime labeled with ^{32}P-dCTP using the Multiprime labeling kit according to the directions of the manufacturer (Amersham Life Science), and unincorporated ^{32}P-dCTP was removed using Sephadex G-25 columns (Boehringer Mannheim). 2 x 10^6 cpm/ml of either the TNFα, p55 or p75 TNF receptor cDNA probes were then added to the hybridization buffer (the composition of the hybridization and prehybridization buffers were identical), and the probes were denatured by boiling for 5 minutes. The probes were then added to their respective Northern blots and hybridization was allowed to proceed overnight at 65°C. The blots were washed twice in a buffer of 40 mM Na phosphate, pH 7.2, 0.5% (w/v) BSA, 5% (w/v) SDS, and 1 mM EDTA for 20 min at 65°C and twice in a buffer of 20 mM Na phosphate, pH 7.2, 1% (w/v) SDS, and 1 mM EDTA for 20 min at 65°C. They were then exposed to film (Kodak X-Omat AR, Eastman Kodak, Rochester, NY) at -80°C and subsequently placed into PhosphorImager cassettes (Molecular Dynamics, Sunnyvale, CA) at room temperature. Quantifications from the PhosphorImager exposures were performed using

ImageQuant software (Molecular Dynamics). Hybridization with both the GAPDH and cyclophilin (p1B15) cDNA probes was used for normalization of loading.

The positive control for TNFα mRNA expression was total RNA isolated from 5 x 10^8 J774A.1 mouse macrophage cells which had been treated for 3 hours at 37°C with 10 ng/ml lipopolysaccharide (LPS B *E coli.* 0111:B4, Difco) according to the procedure of De et al. (34). These cells were cultued in DMEM containing 10% (v/v) FBS and 50 μg/ml gentamicin and passaged every 3.5 days. As a positive control for the TNF receptor Northern blot analysis, TNF receptor mRNA expression was examined in total RNA isolated from 9 x 10^7 Wehi-164 cells, which had previously been shown to contain both p55 and p75 TNF receptor mRNA transcripts (30). The culture medium and passage of these cells is discussed in a forthcoming section.

TNFα and casein Western blot analyses

Proteins from the Trizol homogenates of the MEC organoids isolated from virgin, pregnant, lactating, and post-lactational rats were precipitated with isopropanol (0.8 ml per ml Trizol), centrifuged at 12,000 x*g* for 10 minutes at 4°C, and washed 3 times with 0.3 M guanidine-hydrochloride in 95% (v/v) ethanol and once in 95% ethanol according to the manufacturer's directions. The protein pellets were dried for 10 minutes and solubilized in 10 M urea containing 50 mM DTT for 1 hour, boiled for 4 minutes, sonicated (10 short bursts with a Tekmar Sonic Disruptor), diluted with 4-fold concentrated Laemmli sample buffer (35) and reboiled for 4 minutes. The proteins could then be stored at -20°C. Fifty micrograms (for TNFα) or 2.5 μg (for casein) of total protein (as determined following Biorad protein assay) (36) was electrophoresed on either 15% (TNFα) or 12.5% (casein) polyacrylamide-SDS separating gels (with 4% stacking gels) according to the method of Laemmli (35) in a buffer of 124 mM Tris, 959 mM glycine, 17 mM SDS, pH 8.3. After the gels were equilibrated in transfer buffer (25 mM Tris, 192 mM glycine, 20% (v/v) methanol, pH 8.3) for 30 minutes, the proteins were electrophoretically transferred to nitrocellulose membranes (90 volts for 90 minutes at 4°C), and the membranes were blocked overnight at 4°C with either 5% (w/v) (TNFα) or 1% (w/v) (casein) Blotto in TBS buffer (150 mM NaCl, 10 mM Tris, pH 7.4 at 25°C). Membranes were rinsed in TBS containing 0.1% (v/v) Tween 20 (TBS/Tween) and incubated for 2 hours at room temperature with rabbit polyclonal antiserum against either murine TNFα (Genzyme catalog # IP-410, 1:100 dilution) or the rat casein proteins (27) (1:2000 dilution) in TBS containing 0.5% (w/v) BSA. The blots were washed 5 times for 2 minutes each in TBS/Tween, incubated with biotin-labeled goat anti-rabbit IgG (1:1000 dilution) for 90 minutes at room temperature, washed again, and incubated for 30 minutes at room temperature with streptavidin peroxidase (1:400). The membranes were then developed using the enhanced chemiluminescence system (ECL; Amersham) and/or DAB. (Twenty milligrams of DAB was dissolved in 100 ml of a buffer containing 10 mM Tris, 150 mM NaCl, 1 mM EDTA and 0.1% (v/v) Triton X-100. Immediately before use, 10 μl of 30% H_2O_2 was added. To stop color development, the blots were rinsed in deionized water.)

For the analysis of casein protein accumulation by MEC in primary culture, the sample preparation is described in a forthcoming section entitled *Assessment of MEC functional differentiation (casein analysis)*.

Analysis of p55 TNF receptor protein expression in MEC

MEC organoids were isolated from rats on either day 5 or 10 of lactation as previously described. After isolation, the MEC were lysed in PBS containing 1% (v/v) Triton X-100, 1 mM PMSF, 10 μg/ml leupeptin, 5 mM iodoacetamide and 0.01 μM aprotinin (3.0 ml per 2 x 10^8 cells), sheared through a 22 gauge needle and sonicated 10 seconds on ice. These MEC lysates were incubated with 100 μl of protein A Sepharose for 1 hour at 4°C, after which the Sepharose beads were removed by centrifugation at 1000 x *g* for 5 minutes. Twenty micrograms of either antibody 55R-170.1.89 or 55R-593.4.48 against the murine p55 TNF receptor was then added to each MEC lysate, and the samples were incubated overnight at 4°C with rotation. One hundred microliters of a protein A Sepharose slurry was added and the samples were further incubated for 4 hours at 4°C with rotation. The Sepharose beads were pelleted by centrifugation at 1000 x *g* for 5 minutes and washed three times with 1 ml of immunomix (PBS containing 1% (w/v) SDS, 1% (v/v) Triton X-100, 0.5% (w/v) Na deoxycholate, 1.0 % (w/v) BSA, 3 mM Na azide, 1mM PMSF, 5 mM iodoacetamide, 10 μg/ml leupeptin, and 0.01 μM aprotinin) and once in PBS. The Sepharose beads were then resuspended in 100 μl of 2-fold concentrated Laemmli sample buffer *without* β-mercaptoethanol or DTT (ie: non-reducing) and boiled for 10 minutes. After centrifugation at 1000 x *g* for 5 minutes at 4°C, the supernatant was removed from the beads and stored at -20°C.

For ligand blotting with [^{125}I]-labeled TNFα, 80 μl of each MEC lysate or 40 μg of a rat spleen membrane preparation were loaded onto a 10% polyacrylamide-SDS separating gel (with a 4% stacking gel) and electrophoresed, equilibrated and transferred to a nitrocellulose membrane as described above. The membrane was blocked overnight at 4°C in PBS containing 3% (w/v) non-fat dry milk and 20% (v/v) FBS, rinsed in PBS and incubated with [^{125}I]-labeled TNFα for 4 hours at 4°C. After 3 washes in PBS (2 minutes each), the membrane was exposed to film (Kodak X-Omat AR, Eastman Kodak, Rochester, NY) at -80°C.

For Western blot analysis, 5 μl of each MEC lysate or 40 μg of a rat spleen membrane preparation were loaded onto a 10% polyacrylamide-SDS separating gel (with a 4% stacking gel) and electrophoresed, equilibrated and transferred to nitrocellulose membranes as described above, and the membranes were blocked overnight at 4°C in 3% (w/v) milk in 20 mM Tris, 150 mM NaCl (final pH 7.4). After 4 washes in PBS containing 0.05% (v/v) Tween 20 (2 minutes each), one membrane was incubated with anti-p55 TNF receptor antibody 55R-593.4.48 (2 μg/ml in PBS) for 2 hours at room temperature, while a duplicate membrane was incubated with normal Armenian hamster serum (2 μg/ml in PBS). The blots were washed as just described, incubated with biotin-labeled goat anti-hamster IgG (Caltag; 1:7500 dilution in PBS) for 1 hour at room temperature, washed again, and incubated for 30 minutes at room temperature with streptavidin peroxidase (Zymed; 1:4000 dilution in PBS). The membranes were then developed using the enhanced chemiluminescence system (ECL; Amersham).

Primary culture of MEC

For primary culture studies *in vitro*, MEC organoids from virgin rats were isolated as described above and cultured as previously described (2,26,27). Briefly, after the 4 hour adherence step, the non-adherent MEC organoids were pelleted by centrifugation at 500 x *g* for 10 min and resuspended in ice-cold RBM matrix at a concentration of 3 x 10^5 cells per 0.2 ml. Two hundred microliters of RBM matrix containing MEC organoids was then layered on top of 200 μl pre-gelled, cell-free RBM matrix per well of a 24-well plate and allowed to gel for 3 hours at 37°C in 5% CO_2, 95% air. One milliliter of medium was then added to each well. The medium was changed on day 5 as indicated for each of the experiments below; thereafter, medium was changed twice per week. The complete, serum-free medium contained 10 μg/ml insulin, 10 ng/ml EGF, 1 μg/ml progesterone, 1 μg/ml hydrocortisone, 1 μg/ml prolactin, 5 μg/ml transferrin, 5 μM ascorbic acid, 1 mg/ml fatty acid-free BSA, and 50 μg/ml gentamicin in phenol red-free DMEM-F12 (1:1). Specific culture times and conditions are as indicated below for each series of experiments.

Analysis of individual TNF receptor function in MEC in primary culture

^{3}H-Thymidine incorporation assay. The MEC organoids from virgin rats were cultured until day 5 in suboptimal, low EGF medium, which was identical to the aforementioned serum-free medium except that the EGF concentration was decreased from 10 to 0.1 ng/ml. On day 5, the medium was changed and EGF (10 ng/ml), human TNFα (40 ng/ml; 100 U/ml), murine TNFα (32 ng/ml; 100 U/ml), or various dilutions of the agonistic antibodies specific for either the p55 or p75 TNF receptor were added, and the cultures were incubated for 48 hrs (until day 7) at 37°C in 5% CO_2, 95% air. For the last 4 hours of treatment, the MEC organoids were pulse-labeled with [^{3}H]-thymidine (5 μCi per ml of medium). The medium was then removed and the RBM matrix was digested away using 5 caseinolytic units/ml liquid dispase (1 ml per well) for 2 hours at 37°C. The MEC organoids were pelleted at 500 x *g* for 10 minutes at 4°C, washed once with cold PBS, and repelleted at 1100 x *g* for 10 minutes at 4°C. The acid-insoluble fraction of the MEC organoids was then precipitated overnight at 4°C with 1 ml of 5% (w/v) TCA. The pellets were washed twice with cold 5% TCA, solubilized in 1 ml of 0.1N NaOH containing 0.1% (v/v) Triton X-100 and neutralized with 100 μl of 1N HCl. [^{3}H]-Thymidine incorporation was then determined by liquid scintillation counting.

Morphological analysis. Culture conditions and treatments were as described above for the [^{3}H]-thymidine incorporation assay. The morphological appearance of the MEC organoids was then assessed and quantitated on day 7 of culture (during the last 4-6 hours of the 48 hour treatment) by light microscopic observation. Colonies were classified into four main groups: end bud-like, alveolar, squamous and atypical hybrid. The end bud-like colonies are pale-rust in color, have a more simplistic lobular structure with fewer, smaller ductal projections, and are primarily composed of immature epithelial cells which show little or no evidence of functional differentiation (28). In contrast, the dark brown or black alveolar colonies are larger, have a more complex multilobular structure with extensive ductal projections and are composed of morphologically and functionally differentiated MEC organized into a classical alveolar arrangement (28). The squamous colonies contain concentrically compacted acellular material in a keratotic whorl pattern (2,37), and atypical hybrid colonies are defined as hybrids of alveolar and squamous colonies. Photographs of the organoids were taken with a Nikon FX-35A camera mounted on an Olympus CK2 inverted microscope (Melville, NY).

Assessment of MEC functional differentiation (casein analysis). To assess the effects of the individual TNF receptor agonistic antibodies on MEC functional differentiation, experiments were performed in two different culture conditions. First, to determine if either of the TNF receptor agonistic antibodies was able to stimulate casein accumulation, the MEC organoids from virgin rats were cultured in low EGF medium as described above for the [^{3}H]-thymidine incorporation assay. Under this condition, basal casein accumulation is low, and any increase in casein levels is more easily detected.

In a second series of experiments, MEC organoids were cultured until day 5 in the complete serum-free medium containing 10 ng/ml EGF. This medium stimulates high levels of casein accumulation, so the TNF receptor that signals the TNFα-induced inhibition of casein accumulation can be determined. On day 5 of culture, the medium was changed and human TNFα (40 ng/ml), murine TNFα (32 ng/ml), or various dilutions of the agonistic antibodies specific for either the p55 or p75 TNF receptor were added and the cultures were incubated for 48 hrs (until day 7).

In either case, samples were harvested as previously described for the casein ELISA (2), but casein levels were instead determined using Western blot analysis since the agonistic antibodies against the TNF receptors were found to interfere with the casein ELISA. Briefly, the medium was removed and the gel containing the MEC was scraped from the well and transferred to a microfuge tube on ice. One milliliter of borate buffered saline (BBS), which consisted of 5 parts borate stock buffer (0.1 M boric acid, 0.025 M borax sodium tetraborate and 0.075 M NaCl, pH 8.4 - 8.5) and 95 parts saline (150 mM NaCl) and contained 0.1 mM PMSF, 100 ng/ml soybean trypsin inhibitor, and 20 ng/ml leupeptin, was then added to each sample. The samples were sonicated using a Tekmar sonic disruptor and centrifuged at 12,500 *xg* for 10 minutes at 4°C. The supernate was then aliquoted, snap frozen in liquid nitrogen and stored at -20°C until use. For the Western analysis, the samples from each of the triplicate wells for each treatment group were mixed to create a "representative" sample. Eight micrograms of this "representative" sample (adjusted to equivalent protein content following Biorad analysis) was electrophoresed, blotted and incubated with the polyclonal antibody against the rat casein proteins as described above. The relative intensities of the various casein isoforms on each Western blot were quantified by densitometric analysis using ImageQuant software (Molecular Dynamics).

Analysis of casein protein synthesis

To determine the effects of TNFα and the individual TNF receptor agonistic antibodies on *de novo* casein synthesis, MEC were cultured until day 5 in complete, serum-free medium containing 10 ng/ml EGF. On day 5, the medium was changed and either human TNFα (40 ng/ml) or various dilutions of the TNF receptor agonistic antibodies were added to the complete medium. The cultures were then incubated for 23 hours at 37°C in 5% CO_2, 95% air, at which time the medium was replaced with methionine-free, complete medium containing either human TNFα or the agonistic antibodies. After a 1 hour incubation at 37°C, Tran ^{35}S-label (100 μCi/well) containing ^{35}S-methionine and ^{35}S-cysteine was added, and the cultures were incubated for another 24 hours at 37°C (for a total treatment time of 48 hours). Triplicate samples were then harvested for casein as described in the preceding section. Fifty microliters of each sample was removed and mixed with 50 μl of 10% (w/v) TCA, allowed to sit for 10 minutes on ice, and passed through a GF/A filter; the filter was then rinsed twice with 5% (w/v) TCA, and the amount of incorporated radioactivity in each sample was determined by liquid

scintillation counting of the filter. An equivalent number of (TCA-precipitatable) counts of each remaining sample was then used for the immunoprecipitation of the casein proteins. (To normalize sample volumes, BBS containing protease inhibitor was added to each sample to a final volume of 1 ml.) Each sample was then incubated (with rotation) with the polyclonal anti-casein antibody (1:100 dilution) for 2 hours at 4°C to immunoprecipitate the rat casein proteins. One hundred microliters (0.1 vol) of a protein A Sepharose slurry was added and the samples were further incubated for 30 minutes at 4°C with rotation. The protein A Sepharose beads were then washed three times with TBS, after which an equal volume of 2-fold concentrated Laemmli sample buffer (5046} was added and samples were boiled for 10 minutes. After centrifugation at 750 x *g* for 5 minutes at 4°C, the supernatant was removed from the beads and stored overnight at -20°C. Thirty microliters of each sample was electrophoresed on a 12.5% polyacrylamide-SDS separating gel (with a 4% stacking gel) according to the method of Laemmli (35), and gels were fixed overnight in 4% methanol. To enhance the radioactive signal, the gels were soaked twice in 100 ml of DMSO for 30 minutes each at room temperature and immersed in 20 ml of 20% (w/v in DMSO) PPO for 3 hours. Gels were then soaked in 100 ml of water for 1 hour, dried, and exposed to film at -70°C for 6-10 days.

Effects of the EGF receptor tyrosine kinase inhibitor PD 158780 on TNFα-induced growth and differentiation

For these studies, MEC organoids were isolated from virgin rats and embedded within the RBM matrix as previously described. The MEC organoids were then cultured until day 5 in *EGF-free*, serum-free medium containing 10 μg/ml insulin, 1 μg/ml progesterone, 1 μg/ml hydrocortisone, 1 μg/ml prolactin, 5 μg/ml transferrin, 5 μM ascorbic acid, 1 mg/ml fatty acid-free BSA, and 50 μg/ml gentamicin in phenol red-free DMEM-F12 (1:1). On day 5, the medium was changed and either EGF (10 ng/ml) or TNFα (2 or 40 ng/ml) in the presence or absence of PD 158780 (0.5 μM in 0.1% DMSO) were added, and the cultures were incubated for either 48 hrs (until day 7) or 16 days (until day 21) at 37°C in 5% CO_2, 95% air. After the initial medium change on day 5 for the groups cultured until day 21, fresh medium was thereafter added twice per week. All media containing either TNFα and/or PD 158780 were prepared immediately before feeding. As a control for all of these studies, PD 158780 alone was added to the EGF- and TNFα-free culture medium.

[^{3}H]-Thymidine incorporation assay. For the last 4 hours of incubation on either day 7 or day 21, the culture were pulse-labeled with [^{3}H]-thymidine (5 μCi per ml of medium), and [^{3}H]-thymidine incorporation was determined as described above.

Quantitation of cell number (MTT assay). Cell number was quantitated on days 7 and 21 using the MTT assay that we have previously described (2). In this assay, viable cells convert the soluble tetrazolium MTT dye into insoluble blue formazan crystals. Briefly, 200 μl of MTT (5 mg/ml in PBS) was added per ml of medium, and cultures were incubated for 16 hrs at 37°C. After removal of the medium and rinsing of each well with 1 ml of PBS, the RBM matrix was digested by adding 1 ml of dispase (5 caseinolytic units/ml) per well and incubating for 2 hrs at 37°C. The digested material was then transferred to glass tubes, and each well was rinsed with 1 ml of PBS. The cells were then separated from the digested matrix by centrifugation at 4500 x *g* for 20 minutes at 25°C. The supernatant was discarded, and the pellet was dissolved in 2 ml of isopropanol and recentrifuged for 5 minutes. Absorbance at 570 nm was read in a Bio-Tek model EL311 plate reader (Winooski, VT). Standard curves using the newly isolated MEC were set up for each experiment.

Morphological analysis. The morphological appearance of the MEC organoids was assessed and quantitated on days 7, 14 and 21 of culture by light microscopic observation as previously described.

Assessment of functional differentiation (casein analysis). Casein samples were harvested as previously described on days 7 and 21 of culture. Casein levels were assayed in three wells per treatment group by Sue Shoemaker using the standard casein ELISA previously developed by our laboratory (27). In addition, casein accumulation by MEC was determined using a modification of the aforementioned Western blot procedure. In this case, samples were mixtures of the triplicate samples for each treatment group. The samples were normalized for loading by either equivalent protein contents (following Biorad protein analysis) or based on equivalent cell number as noted in the text. In either case, the sample preparation, gel-electrophoresis and transfer procedures were performed as described in a preceding section. After the membranes were blocked overnight at 4°C with 5% (w/v) Blotto (nonfat dried milk) in TBS buffer (150 mM NaCl, 10 mM Tris, pH 7.4 at 25°C), they were rinsed in TBS containing 0.5% (w/v) BSA (TBS/BSA) and incubated for 2 hours at room temperature with a rabbit polyclonal antibody against the rat casein proteins (27) (1:3000 dilution) in TBS/BSA. Blots were then washed in TBS/BSA five times for 2 minutes each, incubated with peroxidase-conjugated donkey anti-rabbit IgG (1:5000 dilution in TBS/BSA) for 60 minutes at room temperature, washed again five times (5 minutes each) in TBS/BSA containing 0.01% (v/v) Tween 20, and developed using the enhanced chemiluminescence system (Amersham).

Analysis of bioactive TNFα levels in whole mammary glands

The level of biologically active TNFα in extracts of whole mammary glands was assayed using a modified version of the Wehi 164 fibroblast cytotoxicity assay (38). The Wehi-164 cells were cultured in RPMI-1640 containing 10% (v/v) FBS, 3 μg/ml gentamicin and 2 mM glutamine and were passaged every 3.5 days. Mammary glands were excised from virgin, mid-pregnant (day 13-14), lactating (days 5, 10 and 15) or post-lactational (day 7 of involution) female rats and homogenized in RPMI-1640 containing 2mM glutamine (1 ml per 100 mg tissue) using a PCU-2-110 Polytron (Brinkmann Instruments). The homogenate was then centrifuged at 1000 x *g* for 30 minutes at 4°C, followed by centrifugation of the supernate at 13,000 x *g* for 20 minutes at 4°C. The mammary gland extracts were then filtered through a 0.45 μm filter for sterilization. The samples were serially diluted in RPMI-1640 containing 2 mM glutamine and each dilution was plated in quadruplicate. One hundred microliters of Wehi 164 cells (5×10^4 cells) in culture medium (RPMI-1640 containing 10% (v/v) FCS, 2mM glutamine, and 0.003 mg/ml gentamicin) were then added into each well of a 96 well plate in the presence of 0.5 μg/ml actinomycin D (Sigma, St. Louis, MO) to inhibit protein synthesis and facilitate the cytotoxic response to TNFα. After incubation for 22 hours at 37°C, 180 μl of supernatant was removed from each well and replaced with 180 μl of fresh culture medium containing 0.5 μg/ml actinomycin D. Twenty microliters of MTT (5 mg/ml) (Sigma, St. Louis, MO) was then added to every well. After further incubation at 37°C for 4 hours, 150 μl of supernatant was removed from every well, and 100 μl of 0.04 N HCl in isopropanol was added to each well. Plates were then wrapped in aluminum foil with damp paper towels and stored overnight at room temperature. Absorbance at 570 nm was determined using a Bio-Tek (Winooski, VT) model EL 311 plate reader. A standard curve ranging from 0.01 to 1000 U/ml TNFα was set up for each experiment and used to calculate the exact concentration of TNFα in each sample.

TNFα expression in DMBA- and NMU-induced mammary tumors

Mammary carcinomas were induced in virgin, 50- to 60-day-old pathogen free female Sprague-Dawley Crl:CD BR rats purchased from Charles River (Raleigh, NC) and were generously provided by Dr. Clement Ip. Tumors were generated by administration of 9,10-dimethyl-1,2-benz-anthracene (DMBA) (Sigma, St. Louis, MO) (10 mg per rat in 1 ml corn oil) p.o. using standard protocols (39) or via i.p. injection of 1-methyl-1-nitrosourea (NMU) (Ash Stevens, Inc., Detroit, MI) (10 mg/rat in 0.5 ml saline). Animals were palpated weekly to determine the size and location of tumors, and the experiment was terminated 20 weeks after carcinogen administration. Tumors (approximately 1 cm in diameter) were excised from the mammary glands, cleared of surrounding tissue, rinsed in phosphate-buffered saline, quick-frozen in liquid nitrogen and stored at -80°C until use. Four tumors of each type were pooled, pulverized in liquid nitrogen at 4°C, and homogenized in Trizol (1ml per 100mg tumor) using a PCU-2-110 Polytron (Brinkmann Instruments). Total cellular RNA and protein were then prepared following the standard protocol for Trizol, and poly A^+ mRNA was subsequently fractionated using the PolyATract mRNA Isolation System (Promega). Northern blot analysis for TNFα and TNF receptor mRNA and Western blot analysis for TNFα were performed as described above in a previous section. As a control for these experiments, MEC were isolated from age-matched, virgin rats that had not been given carcinogen.

Effects of TNFα on the growth and differentiation of NMU-initiated and transformed MEC

For these studies, initiated and transformed MEC were induced using 1-methyl-1-nitrosourea (NMU) via the method of Thompson *et al.* (40). In brief, NMU was dissolved immediately prior to use in 0.9% saline which had been acidified to pH 4.0 with acetic acid. Within 30 minutes, 21 day old virgin female rats were given injections of 50 mg NMU/kg body weight i.p.; control animals were given vehicle. Animals were then weighed and palpated weekly to determine the incidence, size and location of tumors. Control and NMU-initiated mammary glands were excised from 56 day old animals, while tumors were excised thereafter as they reached approximately 1 cm in average diameter (corresponding age-matched control animals were also sacrificed at the same times as tumor bearing animals). MEC organoids were then isolated from control glands, NMU-initiated glands and NMU-tumors and embedded within the RBM matrix as previously described. The control, initiated and transformed MEC organoids were cultured in *EGF-free*, serum-free medium (as described above) in either the presence or absence of TNFα (4 or 40 ng/ml). In addition, the tumor-derived (and corresponding control) MEC organoids were also cultured in the complete medium (containing 10 ng/ml EGF) in either the presence or absence of TNFα (4 or 40 ng/ml). Fresh medium was added twice per week.

Quantitation of cell number (MTT assay). Viable cell number was quantitated on days 0, 4, 7, 11, 15 and 21 using the MTT assay as described above.

Morphological analysis. The morphological appearance of the normal, initiated and tumor-derived MEC organoids was assessed and quantitated on days 13-15 of culture by light microscopic observation as previously described.

Statistics

Statistical significance was determined using a one-way analysis of variance (ANOVA) with the Student-Newman-Keuls test for pairwise multiple comparisons. $P < 0.05$ was judged to be statistically significant.

RESULTS

Detection and regulation of TNFα production by MEC in primary culture

In the first set of studies, the hormonal regulation of TNFα mRNA expression by normal MEC in primary culture was assessed by removing various hormones, one at a time, from the optimal culture medium, and then determining mRNA expression by Northern blot analysis. Unfortunately, it was discovered that the oligonucleotide probe used in the Northern blot procedure at this time was binding non-specifically to the 28S RNA. Thus, results obtained using this probe were invalid. After a cDNA probe specific for rat TNFα was then procured, we determined that TNFα mRNA is expressed by the epithelial cells of the mammary gland; however, we also discovered that TNFα RNA levels were below the limits of detection using total cellular RNA, and that poly A+ mRNA must be used for Northern analysis. Unfortunately, because of the extremely large numbers of primary MEC required to obtain sufficient amounts of total and poly A+ mRNA, it was not possible to examine TNFα mRNA expression by MEC in primary culture. Thus, we focused our investigations on determining the expression patterns of TNFα and its receptors by normal MEC at the various stages of *in vivo* mammary gland development.

TNFα and TNF receptor expression are specifically and independently regulated in MEC during normal mammary gland development

Previous studies in our laboratory demonstrated that TNFα could serve as a growth and differentiation factor for MEC in culture (2) and suggested that TNFα may play a physiological role in directing the overall growth and development of the mammary gland *in vivo*. If so, it would be logical for cells within the mammary gland to produce this cytokine; therefore, studies were undertaken to determine whether mammary *epithelial* cells express TNFα mRNA. Since various cells located within the mammary stroma, such as adipocytes and fibroblasts, may also produce TNFα, it was first necessary to separate the MEC from the other elements of the mammary gland. Figures 1 and 2 demonstrate that the MEC organoids used to assess the steady state expression levels of TNFα and its receptors were truly representative of epithelial cell organoids at the various *in vivo* stages of development. As can be seen in Figure 1, the organoids are free of loosely associated stromal cells and connective tissues. Moreover, cross-sectional analysis of the epithelial organoids demonstrated that the structures were composed of well polarized columnar epithelial cells organized in a classical mammary-specific pattern (Verstovsek, Darcy and Ip, unpublished observations). MEC organoids isolated from the glands of pubescent, virgin rats were pale-colored, end bud-like ductal structures (Figure 1A) which did not express measurable amounts of casein (Figure 2). As the mammary gland then developed during pregnancy (Figure 1B), the MEC differentiated into dark-colored, multilobular alveoli which had already begun to accumulate significant amounts of the α, β, and γ forms of casein (Figure 2). This alveolar structure was maintained throughout lactation; however, the organoids were slightly lighter in color due to distention with milk (Figures 1C-1F). As expected, casein levels peaked at this time (Figure 2). After one week of involution, the MEC organoids were smaller and darker, but still retained most of their alveolar appearance (Figure 1G). By this time, casein production had essentially ceased (Figure 2).

<u>TNFα expression in normal MEC during puberty, pregnancy, lactation and involution</u>
After confirming that the isolated MEC organoids were free of stromal cells and were morphologically and functionally representative of their respective developmental stages, TNFα

mRNA expression during the various stages of mammary gland development was investigated. As can be seen in the Northern blot in Figure 3A, a single 1.9 kb TNFα mRNA transcript was detected in MEC from all stages of development. As a positive control, total RNA from lipopolysaccharide-stimulated J774A.1 macrophage cells was also analyzed and found to express a similar TNFα mRNA species of 1.9 kb as previously reported (41) (data not shown). Normalization of TNFα mRNA to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) indicated that TNFα mRNA levels dramatically increased during mid-pregnancy and then steadily declined through lactation and involution; however, it should be noted that expression remained elevated relative to TNFα mRNA levels during puberty (Figure 3B).

Since there appeared to be some variation in GAPDH levels even though equivalent amounts of mRNA were thought to have been loaded for each sample, the reasons underlying this variation were investigated. First, prior to isolation of poly A^+ mRNA, the integrity of the 18S and 28S RNA bands was examined in each sample, and the RNA was found to be highly degraded during lactation (data not shown). Thus, it was believed that the variation in GAPDH was reflective of an inability to accurately quantify the RNA prior to loading which then resulted in variations in the amount of RNA loaded per lane. In addition, previous studies have reported that GAPDH expression levels are relatively constant during development (42). To confirm that the changes in TNFα mRNA expression during mammary development were true, the TNFα Northern blots were stripped and re-probed with a second, constitutively expressed housekeeping gene, cyclophilin (p1B15) (31). As is shown in Figure 3C, normalization of TNFα mRNA levels to cyclophilin was equivalent to normalization to GAPDH. Lastly, Northern blot analysis with several, independent sets of mRNA confirmed these changes in TNFα mRNA expression during mammary development .

In conjunction with mRNA expression, TNFα protein levels were also examined in MEC to determine whether mRNA expression was indicative of TNFα protein levels. The 17-kDa soluble form of TNFα was barely detectable and did not appear consistently between experiments. This was not unexpected, however, because these studies were performed on cell extracts, and the soluble form of TNFα would have been lost during the digestion of the gland and/or subsequent washes and filtration of the organoids. The major form of TNFα detected in MEC was the 26-kDa transmembrane cytokine (Figure 4). Although undetectable in MEC from virgin rats, expression of this protein became apparent during pregnancy, and, in contrast to its message, was highest during lactation, where it remained at a relatively uniform, peak level before disappearing during involution. The band at 25-kDa, immediately below the 26-kDa band for TNFα, may be TNFβ, which shows significant homology to TNFα; alternatively, the doublet could represent native and post-translationally modified forms of TNFα (43).

TNF receptor expression in normal MEC during mammary gland development Previous studies by the laboratory which demonstrated that TNFα could affect MEC growth and differentiation in primary culture suggested that TNF receptors were present on normal mammary epithelial cells. Northern blot analysis showed that MEC expressed mRNA transcripts for both the p55 and p75 TNF receptors, and the mRNA levels of each were independently and specifically regulated during puberty, pregnancy, lactation, and involution. For the p55 TNF receptor, a single 2.3 kb mRNA species was detected in MEC from all stages (Figure 5A). As a positive control, total RNA from Wehi-164 cells was also analyzed and found to express a single p55 mRNA transcript of the same size (data not shown). Upon normalization to GAPDH, p55 mRNA was found to increase steadily through pregnancy and into lactation, peak on day 10 of lactation, and then rapidly decrease through involution (Figure 5B). In contrast, MEC from all stages of development expressed three distinct mRNA

transcripts of 2.8, 4.3 and 5.7 kb for the p75 receptor (Figure 6A), and normalization of p75 mRNA expression to GAPDH revealed a modest increase during pregnancy followed by a marked and sustained elevation throughout lactation and involution (Figure 6B). Analysis of total RNA from Wehi-164 cells identified a 3.6 kb transcript for the p75 TNF receptor as previously reported (30) (data not shown). Northern hybridization analysis with several separate sets of mRNA confirmed these developmentally regulated expression patterns for both receptors. Intriguingly, it should be noted that p75 mRNA remained high at the end of lactation and during involution, whereas both TNFα and p55 mRNA levels were decreased at these times.

Investigation of p55 TNF receptor protein expression in normal MEC As for TNFα, studies were also undertaken to assess the profile of TNF receptor protein expression on MEC during mammary gland development. Due to the fact that p55 mRNA expression levels were higher than p75, and because the p55 receptor is often preferentially expressed by cells of epithelial origin (12,44), initial efforts focused on the detection of the p55 TNF receptor. These studies were performed using a series of hamster monoclonal antibodies from Dr. Robert Schreiber which had been generated against the murine p55 TNF receptor (45). First, Western blot analysis was performed on the membrane protein fractions of both rat MEC (from days 5 and 10 of lactation) and rat spleen (as a control for TNF receptor expression) with antibody 55R-593.4.48 (45). No specific bands were identified (data not shown). Next, the p55 receptor was immunoprecipitated from a MEC extract (from day 5 of lactation) via the method of Sheehan (45) with another antibody from Dr. Schreiber, 55R-170.1.89, and attempts were made to identify the receptor using ligand blotting with ^{125}I-TNFα according to the method of Grazioli et al. (46). Even after several different concentrations of the ligand were used, no proteins were identified (data not shown). Lastly, several attempts were made to immunoprecipitate the p55 receptor from MEC extracts (from days 5 and 10 of lactation) with both of the aforementioned anti-p55 TNF receptor antibodies and to subsequently identify the p55 receptor via Western blot analysis with a third antibody from Dr. Schreiber, 55R.286.9.3G10 (45). A band at approximately 55-kDa was identified; however, Western blot analysis of these same samples with normal Armenian hamster serum identified the same protein, thus demonstrating that it was non-specific (Figure 7). Further studies with this antibody were considered futile. Another monoclonal anti-p55 TNF receptor antibody (htr-9, against the human p55 TNF receptor) (47) was obtained from Dr. M. Brockhaus; this antibody failed to react with any proteins in the extracts of either rat MEC (from days 5 and 10 of lactation) or rat spleen (data not shown). Due to the inability to detect the p55 receptor, either because of its low levels or due to a lack of cross-reactivity of these antibodies with rat cells, no attempts were made to identify the p75 receptor via immunoprecipitation and/or Western blot analysis since it was believed to be expressed at even lower levels than p55.

As a final attempt to profile TNF receptor expression on normal MEC, Scatchard analysis was performed on isolated MEC from mid-lactation using ^{125}I-TNFα; MEC from mid-lactation were chosen because the levels of all TNF receptor mRNA transcripts were highest at this time. The results of two experiments were inconclusive (data not shown). Since the process of iodination of TNFα is technically difficult, one possible explanation for the inconclusive Scatchard results may be that the TNFα obtained from DuPont was not properly radio-labeled.

TNFα and TNF receptor expression in mammary fibroblasts from pubescent virgin rats

Numerous studies have demonstrated that fibroblasts can produce TNFα (48-50), and several immunohistochemical studies have identified TNFα protein expression in the stroma of both human (51,52) and rat (N. Stangle and M.M. Ip, unpublished observations) mammary tissue. Therefore, a preliminary investigation of TNFα mRNA and protein expression in fibroblasts isolated from the mammary glands of virgin female rats was also performed. In contrast to the normal MEC, Northern blot analysis failed to identify TNFα mRNA expression in this particular isolate of rat mammary fibroblasts (Figure 8), and neither the 26-kDa transmembrane TNFα protein nor the 17-kDa soluble cytokine were detectable by Western blot analysis (data not shown). It must be considered, however, that the MEC were freshly isolated, while the fibroblasts had been passaged in culture. Even though this culture period was minimal (3 passages), it may have affected the synthesis and/or stability of TNFα mRNA.

In conjunction with these studies of TNFα expression, TNF receptor mRNA expression was also examined in the mammary fibroblasts. As for the normal MEC, a single 2.3 kb mRNA transcript was detected for the p55 TNF receptor in the rat mammary fibroblasts (Figure 9). The mammary fibroblasts also expressed the same three p75 mRNA transcripts as the MEC (Figure 10). Since analysis of TNF receptor protein expression was not successful in the normal MEC, no attempts were made to determine TNF receptor protein expression in the mammary fibroblasts.

TNFα protein expression and bioactivity in whole mammary glands

Although the previously described studies indicated that mammary epithelial cells produce TNFα mRNA and protein, it is not known whether mammary-derived TNFα is physiologically active. In addition, the 17-kDa soluble form of TNFα should, if present, be detectable in homogenates of whole mammary glands rather than in isolated cell extracts. Studies were therefore undertaken to compare TNFα protein expression in whole mammary glands with that in isolated MEC, and to determine the level of biologically active TNFα produced by the mammary gland. When mammary gland extracts from virgin, mid-pregnant, lactating and post-lactational rats were analyzed in a modified Wehi 164 cytotoxicity assay (established by Ignatowski et al. (38)), bioactive TNFα levels were found to be very low (< 0.5 U/ml) with no significant changes during development.

TNFα protein expression was then examined in these same mammary gland extracts. The 17-kDa soluble form of TNFα was not detectable in whole mammary gland extracts at any stage of development (Figure 11); this was somewhat unexpected, as it was believed that examination of whole glands would facilitate the detection of the soluble cytokine. Expression of the 26-kDa transmembrane form of TNFα followed the same pattern as in isolated MEC; the protein was undetectable in mammary glands from virgin rats, became apparent during pregnancy, peaked during mid-lactation and again disappeared during involution. However, the overall level of this protein appeared to be lower in whole mammary glands than in isolated MEC. This apparent decrease may reflect the presence of non-TNFα producing cells in the mammary stroma; however, it should also be noted that different methods were used to prepare the MEC and whole gland samples. The proteins used for analysis of TNFα expression in isolated MEC were derived from Trizol homogenates, while the whole mammary glands were homogenized in RPMI-1640 tissue culture medium containing glutamine. In

contrast to the isolated MEC, a very distinct 32-kDa protein was expressed in whole gland extracts throughout development except for day 21 of lactation, at which time this protein was barely detectable. This band may represent a high molecular weight aggregate form of TNFα; however, it is not believed to be physiologically significant because its expression did not change during the course of mammary development.

Determination of the specific functions of the individual TNF receptors in normal MEC in primary culture

The studies presented above, in conjunction with previous studies by our laboratory on the effects of TNFα in normal MEC, indicated that TNF receptors were present on normal MEC. To determine the specific functions of each TNF receptor in MEC, experiments were performed on MEC organoids in primary culture using agonistic antibodies that were specific to either the p55 or p75 TNF receptor (15). Using a [^{3}H]-thymidine incorporation assay, the ability of each individual TNF receptor antibody to stimulate cellular proliferation was determined. Under suboptimal (0.1 ng/ml EGF) medium conditions, EGF, human TNFα, and the 1:1000 dilution of the p55 antibody significantly stimulated thymidine incorporation by MEC approximately 3-fold, while neither the three higher dilutions of the p55 antibody nor the p75 antibody had any effect (Figure 12); an equivalent concentration of murine TNFα (100 U/ml; 32 ng/ml) had the same stimulatory effect on thymidine incorporation as human TNFα (data not shown). The inability of the p75 antibody to affect MEC organoid growth was not due to a lack of activity, as it was found to stimulate rat thymocyte proliferation (Varela, L.M., M.J. Ehrke and M.M. Ip, unpublished observations).

The effects of each of the TNF receptor agonistic antibodies on the morphological development of the MEC in culture were also determined. Organoids cultured in the presence of 0.1 ng/ml EGF were small, pale-rust in color and had few, if any, small ductal projections. When added into this suboptimal medium, both EGF (10 ng/ml) and human TNFα (40 ng/ml) were able to stimulate classical lobulo-alveolar and branching morphogenesis. After a 48 hour treatment with either of these factors, the organoids were significantly larger and darker in color and networks of ductal projections had begun to emanate from the colonies. Intriguingly, both TNF receptor agonistic antibodies were also able to stimulate alveolar and ductal morphogenesis in a concentration dependent manner. Colonies treated with the 1:1000 dilution of each antibody for 48 hours were similar in appearance to organoids treated with either TNFα or EGF (data not shown). Morphology was not quantitated for the group treated with murine TNFα (32 ng/ml); however, MEC treated with murine TNFα exhibited no visible difference from MEC treated with human TNFα.

To determine how signaling via each individual TNF receptor affected the functional differentiation of MEC in culture, the effects of each TNF receptor agonistic antibody on casein accumulation by the MEC organoids were measured using Western blot analysis; ELISA analysis of these samples was not possible because the anti-TNF receptor antibodies were found to interfere with this assay. In suboptimal (0.1 ng/ml EGF) medium, the addition of EGF (10 ng/ml) increased the accumulation of α_2, β and γ casein, while human TNFα (40 ng/ml) had the opposite effect, decreasing the levels of α_2, β and γ casein isoforms (Figures 13A and 13B). Surprisingly, the p55 and p75 TNF receptor antibodies were found to have opposing effects on functional differentiation. The levels of α_2, β and γ casein were slightly decreased after treatment with the 1:1000 dilution of the p55 receptor antibody, but cells treated with either the $1:10^4$ or $1:10^5$ dilutions of the p75 antibody showed an increase in α_2, β and γ casein accumulation.

In optimal (10 ng/ml EGF) medium conditions, human TNFα (40 ng/ml) decreased the accumulation of the α_2, β, and γ forms of casein by MEC (Figure 14A, compare first and second lanes); murine TNFα (32 ng/ml) had the same inhibitory effect on casein accumulation (data not shown). As under reduced EGF medium conditions, the two TNF receptors had opposing effects on functional differentiation, with a slight inhibition of casein accumulation occurring in response to the p55 antibody and stimulation by the p75 receptor antibody. MEC organoids treated with the $1:10^3$ dilution of the p55 receptor antibody showed a decreased accumulation of β and γ casein (Figure 14A, compare lane 3 to lane 1, and Figure 14B), albeit to a lesser extent than TNFα, whereas treatment with the $1:10^4$ and $1:10^5$ dilutions of the p75 antibody increased the accumulation of α_2 and γ casein by the MEC organoids (Figure 14A, compare seventh and eighth lanes to lane 1, and Figure 14B).

In light of the unexpected discovery that signaling via the two TNF receptors had opposing effects on casein accumulation by MEC in primary culture, it was decided to investigate the potential mechanisms whereby these receptors may be mediating their contrasting effects. The two receptors could independently regulate casein gene transcription, modulate messenger RNA stability, and/or alter the rate of casein protein synthesis, degradation or secretion. An initial study was therefore undertaken to assess the effects of the individual TNF receptor antibodies on casein protein synthesis. Immunoprecipitation of the ^{35}S-methionine labeled casein proteins showed that the p55 and p75 TNF receptor antibodies also had opposing effect on casein synthesis after 48 hours. The 1:1000 dilution of the p55 receptor agonistic antibody inhibited the synthesis of α_1, α_2, and γ casein, while the p75 antibody stimulated the synthesis of all casein isoforms in a concentration-dependent manner (Figure 15). It should also be noted that the *synthesis* of α_1 was markedly affected although its accumulation was not, and that the p75 antibody induced a very pronounced increase in the synthesis of all casein isoforms.

Investigation of the role of the EGF receptor in TNFα action in normal MEC

The aforementioned studies identified the particular functions mediated by each TNF receptor in normal MEC; however, the postreceptor pathways whereby TNFα elicits its effects in normal MEC are still unknown. Previous studies in the laboratory demonstrated that TNFα could replace EGF in the serum-free culture medium used for the MEC and could stimulate MEC growth in a manner identical to EGF (2). Since numerous reports have documented the effects of TNFα on the EGFR in other cell types, and because TNFα can also induce the expression of various ligands of the EGFR, such as TGFα (19,53) and amphiregulin (54), it was postulated that signals from the p55 TNF receptor may up-regulate EGFR ligand production and/or EGFR tyrosine kinase activity to subsequently stimulate MEC growth. To test this hypothesis, we attempted to block the TNFα-induced proliferation of MEC with an inhibitor of the EGFR tyrosine kinase activity, PD158780 (55). This compound has poor inhibitory activity against the PDGF or FGF receptor tyrosine kinases but is a potent inhibitor of the tyrosine kinase activity of members of the EGFR family, which includes the EGFR itself as well as erbB2, B3 and B4. Preliminary studies which evaluated the effects of various concentrations of the EGFR tyrosine kinase inhibitor on MEC indicated that at 0.5 μM, PD158780 was inhibitory to EGF-induced effects but was not cytotoxic to the cells (K.M. Darcy et al., manuscript in preparation). Thus, this concentration of PD158780 was used in an attempt to inhibit the effects of TNFα on normal MEC growth and differentiation.

Effect of PD158780 on the TNFα-induced proliferation of normal MEC First, the ability of PD158780 to block TNFα-induced DNA synthesis in normal MEC was investigated using a ^{3}H-thymidine incorporation assay. When assessed on day 7 of culture after 48 hours of treatment, the EGFR tyrosine kinase inhibitor only modestly suppressed the increase in ^{3}H-thymidine incorporation stimulated by 40 ng/ml TNFα (Figure 16, bars 5 and 6) but reduced DNA synthesis in response to 2 ng/ml TNFα by approximately 50% (Figure 16, bars 7 and 8). In contrast, the EGF-induced increase in DNA synthesis was completely blocked by PD158780 (Figure 16, bars 3 and 4). Moreover, the EGFR tyrosine kinase inhibitor alone was found to cause a 50% inhibition of ^{3}H-thymidine incorporation. Since no EGF was present in the culture medium, we believe that this inhibition was due to the blockage of the activity of endogenous EGFR ligands either produced by the MEC themselves or present in the RBM matrix.

The MEC culture system utilized herein also permits both morphological and functional differentiation over a 3 week period. In order to determine whether the pathway of TNFα action changed as the MEC differentiated, the cells were also treated until the end of the 21 day culture period (for a total of 16 days). When measured on day 21, ^{3}H-thymidine incorporation by MEC treated with either concentration of TNFα was not increased relative to the control as it had been on day 7 (Figures 16 and 17, compare bars 5 and 7 to bar 1). However, it can be seen that PD158780 still had no effect on DNA synthesis in the presence of 40 ng/ml TNFα (Figure 17, compare bars 6 and 5), but again caused a 50% inhibition at the lower, 2 ng/ml concentration (Figure 17, compare bar 8 with bar 7). Of considerable interest was the marked difference in thymidine incorporation between TNFα- and EGF-treated MEC at this time. In contrast to both TNFα-treated groups and to day 7, thymidine incorporation by MEC grown in the presence of EGF for 16 days was dramatically decreased compared to the control group; moreover, PD158780 had no further inhibitory effect on DNA synthesis in the presence of EGF. Also noteworthy was the marked inhibitory effect of PD158780 on thymidine incorporation in the absence of added growth factor (Figure 17, compare bars 2 and 1).

Concurrently, the total, viable cell number was also quantitated in the various treatment groups in order to determine the balance between cell growth and death in response to TNFα, and to determine whether this balance was affected by inhibition of EGFR tyrosine kinase activity. In contrast to the stimulatory effect on thymidine incorporation, neither concentration of TNFα, in either the presence or absence of PD158780, had any effect on viable cell number after 48 hours (day 7). Cell number was increased in the EGF-treated group, however, and this increase was completely suppressed by PD158780, while cell number was unaffected by the 48 hour treatment with PD158780 alone (Figure 18).

When assessed on day 21 after 16 days of treatment, the viable cell number in both TNFα-treated groups was increased when compared to day 7 and was significantly higher than the cell number in the EGF-treated culture (Figure 19, compare bars 5 and 7 to 3). More significantly, PD158780 was unable to block the increase in viable cell number induced by 40 ng/ml TNFα (Figure 19, compare bars 5 and 6), but partially suppressed the increase in cell number in response to 2 ng/ml TNFα. Finally, the EGF-induced increase in viable cell number was blocked by PD158780 (Figure 19) as it had been at day 7, while the EGFR tyrosine kinase inhibitor alone had no effect (compare bars 1 and 2). Thus, when taken together, these data suggest that EGFR tyrosine kinase activity is not required for TNFα to induce the proliferation of normal MEC in culture, although the pathway(s) activated by both the TNFα and EGF receptors may act in concert to stimulate MEC growth under certain circumstances.

Effect of PD158780 on TNFα-induced morphological differentiation We have previously shown that both TNFα and EGF can stimulate the ductal and alveolar morphogenesis of MEC in culture while suppressing the outgrowth of colonies with an atypical or squamous epithelial morphology (2,37). In order to determine whether the EGFR was involved in the pathway by which TNFα stimulated the morphological development of the MEC, the ability of PD158780 to inhibit TNFα-induced morphogenesis was assessed. Morphological changes after 48 hours of treatment were subtle (data not shown). In both TNFα- and EGF-treated groups, the percentage of the less-differentiated, end bud-like colonies was slightly decreased while the percentage of the more-differentiated, alveolar colonies was increased; the percentage of squamous and atypical hybrid colonies in all groups was negligible at this time. The EGFR tyrosine kinase inhibitor had no significant effect on colony morphology in either the presence or absence of TNFα or EGF.

In contrast, TNFα induced significant changes in both colony type and size after 16 days of treatment. Specifically, 40 ng/ml TNFα stimulated the formation of complex, lobulo-alveolar colonies which were significantly larger than those induced by EGF (Figure 20, compare j with f and g) and which were interconnected by extensive ductal branching (Figures 20j and 21). Furthermore, TNFα also inhibited the development of both squamous and atypical colonies (Figure 21). More importantly, the EGFR tyrosine kinase inhibitor was unable to inhibit this expansive TNFα-induced morphogenesis, as colonies that developed in the presence of TNFα plus PD158780 were as large, viable and morphologically developed as those which developed in the presence of TNFα alone (Figures 20j, 20k and 21). The 2 ng/ml concentration of TNFα also stimulated alveolar and ductal morphogenesis, although the effects were less pronounced than those of 40 ng/ml TNFα, and PD158780 was still unable to block this TNFα-induced morphological differentiation ((2) and data not shown).

In contrast to its inability to alter morphological development in the presence of TNFα, the EGFR tyrosine kinase inhibitor had several noteworthy effects on MEC morphogenesis when added alone or in combination with EGF. Unexpectedly, PD158780 alone mimicked the ability of EGF to both suppress squamous and atypical colony formation and to stimulate the formation of the more differentiated, lobular-alveolar colonies (Figure 21). These latter colonies, however, tended to be quite small in comparison to TNFα- or EGF-treated colonies (Figure 20d), and any larger alveolar colonies which developed in the presence of PD158780 alone appeared to be highly disrupted and were surrounded by small cellular bodies which had apparently broken off of the periphery of the alveolar organoids (Figure 20e). Furthermore, organoids treated with both EGF and PD158780 were not as large as colonies treated with EGF alone (Figure 20, compare h and i to g), and many of the alveolar colonies in this group also appeared to be highly disrupted (Figure 20i).

Effect of PD158780 on TNFα-modulated MEC functional differentiation Previous studies by our laboratory determined that TNFα had a complex, biphasic effect on casein production by MEC: a higher concentration of TNFα (40 ng/ml) inhibited functional differentiation, while in the absence of EGF, a lower concentration of TNFα (2 ng/ml) enhanced casein accumulation (2). To determine whether these effects of TNFα might be mediated through the EGFR, the ability of PD158780 to interfere with the effects of TNFα on casein accumulation by MEC was measured using both Western blot analysis and an ELISA (27). When examined on day 7, casein accumulation was found to be unaffected by the 48 hour treatment with either 2 or 40 ng/ml TNFα (Figure 22, lane 5 and data not shown), and casein levels were also unchanged in MEC which had been treated with both TNFα and PD158780

(Figure 22, lane 6 and data not shown). The EGFR tyrosine kinase inhibitor completely blocked the increased accumulation of all casein isoforms in response to EGF (Figure 22, compare lanes 3 and 4), while PD158780 alone had no significant effect on casein levels (Figure 22, compare lanes 2 and 1). Casein levels in all but the EGF-treated group were below the limits of detectability by ELISA (data not shown).

When measured on day 21, however, casein levels were found to be significantly decreased by the 16 day treatment with 40 ng/ml TNFα, and the EGFR tyrosine kinase inhibitor had no effect on this TNFα-induced inhibition (Figure 23A, compare lanes 1, 5 and 6, and Figure 23B). Despite its ability to significantly stimulate both MEC growth and morphogenesis, 2 ng/ml TNFα had no effect on casein levels after 16 days of treatment even though this TNFα concentration had previously been shown to increase casein accumulation by MEC at later times in culture (Figure 23A, lanes 1 and 7, and Figure 23B). Casein levels were also unchanged in MEC which had been treated with both 2 ng/ml TNFα and PD158780.

Unexpectedly, casein accumulation was *increased* in MEC which had been treated for 16 days with PD158780 alone or with both EGF and PD158780 (Figure 23A and 23B). This latter effect was in direct contrast to the inhibitory effect of PD158780 on EGF-induced casein accumulation after 48 hours. Lastly, it should also be noted that this PD158780-induced increase in casein accumulation was not observed in the presence of either concentration of TNFα (Figure 23A and 23B).

TNFα and TNF receptor expression in DMBA- and NMU-induced mammary tumors

TNFα expression in rat mammary carcinomas Since previous studies of TNFα expression in breast cancer have been performed primarily in human tissue, a preliminary study was undertaken in order to determine whether TNFα expression was altered in DMBA- and/or NMU-induced rat mammary carcinomas. Northern blot analysis showed that TNFα mRNA expression was barely detectable in both DMBA- and NMU-induced rat mammary tumors (Figure 24). When these same Northern blots were hybridized with the GAPDH probe for normalization, GAPDH expression levels were also found to be markedly decreased in both tumor types when compared to the age-matched MEC control (Figure 24). In order to determine whether these variations were due to actual changes in GAPDH during carcinogenesis or were the result of RNA degradation, the integrity of the 18S and 28S RNA bands in aliquots of the total RNA for each sample was then analyzed. In this case, the RNA was found to be intact for all samples with no evidence of degradation; however, subsequent normalization of GAPDH expression in these total RNA samples to the ethidium-bromide stained 28S RNA band revealed that GAPDH expression in both tumor types was markedly decreased when compared to normal, age-matched MEC (data not shown). Therefore, TNFα mRNA expression was not normalized directly to GAPDH but was instead equalized to the GAPDH/28S RNA ratio. Even after accounting for the decline in GAPDH expression in the tumors, TNFα mRNA levels were still found to be greatly decreased in both tumor types when compared to normal, age-matched MEC (Figure 24). Furthermore, neither the 26-kDa transmembrane form of the TNFα protein nor the 17-kDa soluble cytokine was detectable in either tumor type. TNFα protein expression was also absent in normal, age-matched MEC from virgin rats (data not shown).

TNF receptor mRNA expression in rat mammary carcinomas Since the aforementioned studies demonstrated that expression of both TNF receptor mRNA transcripts was developmentally regulated in normal MEC, it is also possible that TNF receptor expression may

also vary during mammary carcinogenesis. A preliminary study of TNF receptor mRNA expression in both DMBA- and NMU-induced rat mammary tumors was therefore performed. For the p55 TNF receptor, a single, 2.3 kb mRNA transcript was detected in both DMBA- and NMU-induced mammary carcinomas as well as in normal, age-matched MEC (Figure 25). Normalization of p55 mRNA expression to the GAPDH/28S RNA ratio revealed that p55 TNF receptor mRNA expression was markedly lower in both tumor types than in normal MEC (Figure 25). In contrast, p75 TNF receptor mRNA was not detectable in either tumor type (data not shown). Since Western blot analysis for the p55 TNF receptor was not possible in the normal mammary epithelial cells, no attempts were made to detect TNF receptor protein in either the DMBA- or NMU-induced mammary carcinomas.

Effect of TNFα on the growth and differentiation of initiated and transformed MEC

Effect of TNFα on the proliferation of NMU-initiated and transformed MEC Because previous studies have demonstrated that breast cancer cells in culture are often sensitive to the cytostatic and/or cytotoxic effects of TNFα, we wanted to determine how TNFα affected the growth of both initiated and transformed MEC in primary culture, and to compare these effects with our previous studies in which we determined that TNFα actually stimulates the growth of normal MEC. Thus, MEC were isolated from the mammary glands of NMU-initiated rats or from NMU-induced mammary tumors, placed into primary culture, and treated with TNFα. Over the three week culture period, TNFα was found to stimulate the growth of the NMU-initiated MEC in a manner identical to its growth stimulatory effect on normal MEC (Figure 26).

In contrast, the effects of TNFα on the growth of transformed MEC were more complex. While the normal MEC in either EGF-free or complete (containing 10 ng/ml EGF) medium began to proliferate during the first week of culture, no change in the total *tumor* cell number was observed; TNFα also had no effect on tumor cell number at this time (Figures 27 and 28). Furthermore, in contrast to the significant growth stimulatory effect of TNFα on normal MEC in either medium condition, TNFα had no significant effect on total tumor cell number in EGF-free medium (Figure 27), and only induced a slight increase in tumor cell number in the presence of the complete, serum-free medium (Figure 28).

Effect of TNFα on the morphological development of NMU-initiated and transformed MEC In order to determine whether TNFα could affect the morphological development of either initiated or transformed MEC, the morphological appearance of the organoids in culture was assessed and quantitated over the three week culture period. As for its effect on proliferation, the effects of TNFα on the morphogenesis of NMU-initiated MEC were identical to its effects on the normal MEC (Figure 29, compare d and b, and Figure 30). As previously described, TNFα stimulated the formation of complex, lobulo-alveolar colonies which were interconnected by extensive ductal branching, and inhibited the development of both squamous and atypical colonies.

In contrast, the morphological appearance of the tumor cell organoids was significantly different from the normal MEC. In EGF-free medium, the normal MEC developed into either simple, squamous organoids (Figure 31a) or small, lobulo-alveolar colonies (Figure 31b). Although tumor cells also formed atypical, squamous-like organoids, they tended to be more complex and numerous than their counterparts in normal MEC cultures (Figure 31, compare f to a),; moreover, almost no alveolar colonies developed (Figure 32). Unexpectedly, the tumor-derived organoids in complete medium (containing EGF) also developed into complex, atypical structures (Figure 31i and 31j, and Figure 33) instead of the typical lobulo-alveolar structures formed by normal MEC (Figure 31d). Intriguingly, TNFα was found to induce the

metamorphosis of these atypical tumor cell organoids into multi-lobular alveolar colonies in either EGF-free or complete medium (Figure 31, compare g and h to f, and k to i and j, and Figures 32 and 33); however, TNFα was unable to induce the extensive ductal branching of the tumor-derived MEC as it did in cultures of normal MEC (Figure 31, compare g, h, and k to c and e). Cross-sections of the normal, initiated and tumor-derived organoids have been prepared, and histological analysis is currently underway.

DISCUSSION

Investigation of TNFα and TNF receptor expression and function in normal MEC

The experiments presented herein demonstrate that normal rat MEC produce TNFα and strengthen our previous suggestion that TNFα may play a physiological role in directing the growth and development of the mammary gland. These studies are the first to directly demonstrate TNFα and TNF receptor mRNA expression in freshly isolated, purified normal rat MEC and to obtain sizes for these mRNA species. In addition, the data show direct evidence for the existence of the 26-kDa, membrane-bound form of TNFα in normal MEC. Moreover, these studies also demonstrate that TNFα and TNF receptor expression are independently and specifically regulated during mammary gland development. When taken in conjunction with previous reports by our laboratory (4688}, this suggests that TNFα, acting in concert with various other hormones and growth factors, is a physiological regulator of normal mammary gland development. In contrast to our studies in rats, a study by Pusztai and co-workers (51) failed to detect TNFα or p75 TNF receptors in normal human breast tissue from non-pregnant patients using immunohistochemical techniques, while a separate study using both *in situ* hybridization as well as immunohistochemistry localized both TNFα and TNF receptor expression exclusively to the mammary stroma (52). Basolo and co-workers (56), however, identified TNFα protein expression in alveolar and ductal epithelial cells using immunohistochemistry, and detected TNFα mRNA in isolated, cultured human MEC using reverse transcription-PCR. The differences in expression patterns observed in these various studies most likely result from the use of different methodologies with varying sensitivities. Finally, Dollbaum and co-workers (24), using radioligand binding, demonstrated that TNF receptors were present on normal mammary epithelium but did not determine which specific receptor(s) were present.

Potential roles of TNFα and its receptors in the mammary gland during development

Puberty. Given the relatively high proliferative rate of the MEC at this time, and since TNFα was shown to stimulate the growth and ductal morphogenesis of MEC *in vitro*, it was predicted that TNFα levels would be elevated as the epithelium proliferates and invades the mammary gland fat pad during this developmental stage. Our Northern data, however, indicate that TNFα mRNA levels are low in 50-day old virgin rats, but the possibility that TNFα expression and/or activity was higher in younger rats cannot be excluded. Alternatively, TNFα production may be primarily stromal rather than epithelial at this time. Indeed, immunohistochemical studies of TNFα protein expression in whole glands have identified high levels of TNFα in mammary adipocytes in 50-day old rats (N. Stangle, L.M. Varela, and M.M. Ip, unpublished observations); however, it is not known whether this TNFα protein represents the soluble form of TNFα bound to its receptor(s) or rather reflects an adipocyte-produced, 26-kDa transmembrane form of TNFα. If the adipocytes do produce TNFα, it would then be present to stimulate the growth and branching morphogenesis of the epithelial cells, but its levels would not be elevated in the MEC themselves, which would explain the low levels of TNFα mRNA that were detected in the MEC at this time.

In conjunction with TNFα, both p55 and p75 TNF receptor mRNA expression levels were also low at this time; however, it should be noted that three transcripts were detected in MEC for the p75 TNF receptor during puberty and all other stages of mammary development, while studies of p75 mRNA expression in other cell types have only identified, at most, two p75

transcripts (30,57). It would be interesting to determine whether these transcripts translate into different receptor subtypes on different epithelial cells (ie: ductal versus alveolar), and/or whether they code for different forms of the p75 receptor with unique functions within the mammary gland.

Pregnancy. The pronounced increase in TNFα mRNA during pregnancy suggests a role for TNFα in promoting both the proliferation and morphogenesis of the mammary epithelium during this stage. To support this theory, we have previously demonstrated that TNFα can stimulate MEC proliferation and induce extensive morphological differentiation in primary culture (2). Moreover, p55 TNF receptor mRNA levels significantly increased during pregnancy, and studies of the specific functions of the individual TNF receptors demonstrated that p55 is the sole mediator of the TNFα-induced proliferation of MEC in culture. This latter observation was strengthened by the finding that the antibody directed against the p75 receptor stimulated rat thymocyte proliferation (data not shown), thus demonstrating that the failure of this antibody to stimulate rat MEC DNA synthesis was not due to a lack of functional cross-reactivity with rat cells. Therefore, when taken together, these studies suggest that TNFα-induced growth and morphological development of MEC during pregnancy is signaled primarily via the p55 receptor.

In contrast to the stimulation of growth and morphogenesis during pregnancy, casein milk protein production is largely suppressed until after parturition (58). Previous reports by our laboratory have demonstrated that TNFα (40 ng/ml) reduced casein accumulation by MEC (2), and functional studies of the individual TNF receptors presented herein indicated that signaling through the p55 receptor decreased both casein synthesis and protein accumulation. Thus, in concert with other key regulatory hormones such as progesterone, TNFα may act via the p55 receptor during pregnancy to inhibit casein production until the onset of lactation. It must be noted, however, that only one time point during gestation was examined, so it cannot be ruled out that significant changes in TNFα production and/or function may occur at other times during pregnancy.

Lactation. In contrast to the high levels of TNFα mRNA observed during pregnancy, TNFα mRNA levels steadily declined throughout lactation. It could be argued that this decrease was a dilutional effect due to the onset of high-level milk protein gene expression; however, since the expression patterns of both p55 and p75 TNF receptor mRNA were different from TNFα and from each other at this time, it is believed that the decline in TNFα mRNA levels observed during lactation is specific. In contrast, the 26-kDa transmembrane form of TNFα protein was expressed at its highest levels during lactation, which may reflect either an increase in the half-life of the TNFα protein or a decrease in the cleavage of the membrane form and thus decreased release of the soluble cytokine. Consequently, instead of widespread, systemic action within the mammary gland, as may occur during the proliferative phase of pregnancy, TNFα would only be able to act in a very localized, cell-to-cell manner. Numerous reports have documented the difference in activity between the soluble and transmembrane forms of TNFα (7,8), and the two forms of may also act via different TNF receptors. Thus, expression of transmembrane TNF during lactation, in combination with other hormones and growth factors, may act to stimulate casein production and/or secretion during lactation.

Despite the heightened expression of the 26-kDa form of TNFα at this time, no appreciable levels of biologically active TNFα were detected in whole mammary gland extracts during lactation or any other stage of mammary development. One explanation for this may be

that the level of bioactive TNFα present was simply below the limit of detectability of the cytotoxicity assay. This hypothesis is supported by the fact that it was necessary to use poly A^+ mRNA to detect TNFα expression in MEC, which indicates that TNFα levels in MEC are quite low. Furthermore, the expression levels of the 26-kDa form of TNFα appeared to be lower in the whole gland samples than in the isolated MEC, so TNFα levels may have been "diluted" by the presence of non-TNFα producing cells in the mammary stroma. In contrast, however, there may also be inhibitors of TNFα-induced apoptosis in these extracts which could prevent TNFα from inducing a cytotoxic response in the bioactivity assay.

In contrast to TNFα mRNA expression, p55 mRNA levels continued to increase into early lactation but thereafter declined and remained low into involution. Since the data suggest that TNFα may stimulate growth and inhibit casein via the p55 receptor during pregnancy, and because the transmembrane form of TNFα may preferentially bind and act through p75 (59), p55 may no longer be needed after the proliferative burst in early lactation (60), so its levels may decline. Concurrent with this decrease in p55 mRNA levels, p75 mRNA expression was increased and remained elevated throughout the remainder of lactation, and functional studies showed that signaling via p75 actually stimulated casein synthesis and accumulation by MEC. When considered in conjunction with the aforementioned study by Grell et al. which suggests that the transmembrane form of TNFα acts primarily via p75 (61), it is tempting to speculate that the membrane form of TNFα observed during lactation acts via the p75 receptor to stimulate casein production. Therefore, soluble TNFα may work via the p55 receptor to stimulate growth and morphological development and inhibit casein during pregnancy, while the transmembrane form of TNFα may act via p75 during lactation to stimulate casein production. This disparity in the functions of the two receptors is a well documented phenomenon, and could be due to the differential expression of the two receptors on different cell types (12,62). For example, p55 TNF receptors may predominate on actively dividing epithelial cells within the mammary gland, such as those found in newly branching ducts during puberty and pregnancy, whereas p75 receptors may only be present on the differentiated, milk-producing alveolar cells. Unfortunately, it was not possible to examine TNF receptor protein expression by Western blot analysis due to the low receptor protein levels and/or the inability to obtain an appropriate rat-reactive antibody for Western blotting.

Involution. Since the induction of apoptosis is one of the most well documented functions of TNFα (63), it is possible that TNFα may also play a role in directing apoptosis during mammary gland involution. Although TNFα mRNA levels were not significantly elevated when examined after one week of involution, the possibility that TNFα levels and/or activity were increased during other (especially earlier) stages of involution cannot be ruled out. For example, TNFα levels may have been higher on days 3-4 of involution when the most pronounced regression was taking place.

Nonetheless, it should be noted that the level of TNFα mRNA in MEC during involution was still higher than that in MEC during puberty, and the 26-kDa transmembrane form of the TNFα protein was no longer apparent at this time. Although undetectable by Western blot, this may be reflective of a decreased protein half-life or an increased cleavage and release of the soluble, 17-kDa cytokine, so that instead of localized activity, TNFα may again have widespread, systemic activity throughout the gland. A role for TNFα in apoptosis would be seemingly contradictory to its proposed role as a growth and differentiation factor during mammary gland development; however, it could be hypothesized that an inhibitor of the cytotoxic activity of TNFα may be produced during development. Alternatively, proteins

required for downstream signaling in the pathway whereby TNFα induces apoptosis may only be present during involution.

It is also intriguing that p55 TNF receptor mRNA levels were low at one week of involution while p75 mRNA expression remained elevated at this time. Since the p55 receptor shares the "death domain" region of homology with Fas/APO-1 (64) and because p55 has been shown to be the primary signal transducing receptor for TNFα-induced apoptosis (13,64-66), p55 TNF receptor levels were expected to rise during mammary gland involution. However, p55 receptor levels may have been increased at earlier times during involution, such as days 2-4, when the most pronounced apoptosis and regression of the mammary epithelium were occurring. In contrast, the significance of the persistent elevation of p75 mRNA levels after one week of involution is unclear.

Potential effects of TNFα on the mammary stroma during development. In addition to serving as an autocrine growth factor for MEC during mammary gland development, TNFα may also have significant effects on the mammary stroma. Since other groups have detected TNF receptors on fibroblasts and adipocytes (67-70), these cell types are obvious targets for MEC-derived TNFα. In other systems, TNFα has been shown to stimulate adipocyte lipolysis (71), so TNFα may be a key mediator of mammary adipocyte lipolysis during lactation (72). In contrast, however, TNFα has also been shown to stimulate hepatic lipogenesis (73). Since it is well documented that TNFα serves different roles in different cells, it could be hypothesized that TNFα stimulates lipid mobilization in adipocytes while concurrently stimulating lipogenesis in MEC. Lastly, as our preliminary immunohistochemical data suggests, the cells within the mammary stroma may themselves produce TNFα which could act in either an autocrine and/or paracrine fashion to modulate growth and development.

The EGF receptor is not required for TNFα action in normal MEC

EGFR tyrosine kinase activity is not necessary for TNFα-induced MEC proliferation
The results of our previous studies indicated that the combined mitogenic action of TNFα and EGF on normal MEC was less than additive (2). This suggested that TNFα and EGF might be activating a common growth stimulatory pathway, and that the mitogenic effect of TNFα might be mediated, at least in part, via activation of the EGFR in response to signals from the p55 TNF receptor. On the other hand, this earlier data also suggested that the mitogenic signaling pathway triggered by the p55 TNF receptor may have an EGFR-independent component, since TNFα was shown to increase total cell number to a higher level than that observed with optimal levels of EGF [(2,26,37); Figure 19]. The studies presented herein suggest that EGFR tyrosine kinase activity is not required for TNFα to stimulate MEC proliferation. PD158780 was unable to inhibit proliferation in response to 40 ng/ml TNFα and only partially suppressed the growth promoting effects of 2 ng/ml TNFα. Although we cannot rule out the possibility that PD158780 may be inhibiting an, as yet undiscovered, tyrosine kinase which is activated by 2 ng/ml TNFα, this latter observation suggests that signaling pathway(s) activated by endogenous EGFR ligands may act in concert with pathways activated by the lower concentration of TNFα to stimulate growth. In support of this theory, EGF and TNFα have been shown to preferentially activate different members of the mitogen-activated protein (MAP) kinase family in other cell types. EGF strongly stimulates ERK1 and ERK2 activity, while TNFα induces a more pronounced activation of the c-Jun N-terminal kinases (JNKs) (74-76). Thus, when EGFR activation is blocked by PD158780 such that ERK1 and/or ERK2 may no longer be activated by

this receptor, growth in the presence of 2 ng/ml TNFα may be partially, but not completely, suppressed.

In contrast, the higher (40 ng/ml) concentration of TNFα may be able to directly activate ERK1 and/or ERK2 such that the blockage of endogenous EGFR ligand-induced action by PD158780 has no effect on DNA synthesis in response to this TNFα concentration. Even though TNFα is not as powerful a stimulus for ERK1 and ERK2 as EGF, numerous studies have demonstrated that TNFα can activate these enzymes (77-80), so it could be proposed that the higher concentration of TNFα may activate both the JNK and ERK enzymatic signaling cascades to stimulate MEC growth. Furthermore, a study by Sluss et al. (81) has demonstrated that there are two JNK protein kinase isoforms, JNK1 and JNK2, and that the activation of these protein kinases by TNFα is concentration-dependent. Therefore, it could be postulated that 40 ng/ml TNFα activates both JNK1 and JNK2, while the lower TNFα concentration only triggers the activation of one JNK isoform. The higher TNFα concentration may also preferentially activate JNK2, which has been shown to be more potent in inducing the phosphorylation of c-jun than JNK1 (81). The specific roles of both of the ERK and JNK MAP kinase isoforms in stimulating MEC growth in response to both TNFα and EGF are currently under investigation.

The differential effects of TNFα and EGF on MEC proliferation The distinct effects of TNFα and EGF on MEC proliferation after different times in culture were intriguing. When assessed on day 7, thymidine incorporation, but not cell number, was increased in response to TNFα, while both DNA synthesis and cell number were increased by EGF. This observation further supports our contention that the pathways of TNFα- and EGF-induced growth in normal MEC are different. In addition, this difference in cell number between the TNFα- and EGF-treated groups may be due to the ability of TNFα to increase the overall rate of cell turnover at this time. Since several studies have demonstrated that TNFα can be either cytotoxic or mitogenic to certain cell types (82,83), it is possible that in addition to an overall stimulation of MEC proliferation, TNFα may be cytotoxic to a specific subset of MEC within the cultures, such that there is no net increase in cell number. However, once the MEC to which TNFα is cytotoxic have been eliminated, the proliferative effects of TNFα may become apparent. EGF, in contrast, may have no effect on the rate of cell turnover at this time; thus, its growth stimulatory effects are immediately evident.

In contrast to day 7, the total cell number on day 21 in the groups treated with either 2 or 40 ng/ml TNFα was significantly higher than that in the EGF-treated cultures (Figure 19). Of further interest, however, was the observation that MEC cultured in the presence of either concentration of TNFα were still actively synthesizing DNA at this time (Figure 17), albeit at a reduced level when compared to day 7, whereas DNA synthesis by EGF-treated MEC was significantly lower than the level in TNFα-treated cells. The level of ^{3}H-thymidine incorporation by the EGF-treated cells on day 21 was also significantly lower than the level on day 7 and was actually 3.5 fold lower than thymidine incorporation by MEC cultured in the absence of EGF or TNFα. This latter observation suggests that EGF may be acting to inhibit cell growth at this time; however, the fact that PD158780 did not reverse this inhibition argues against this supposition. Rather, the data suggest a loss of responsiveness of the EGF-treated cells to EGF, perhaps by down-regulation of EGFR activity in response to the 16 day EGF treatment. Alternatively, or perhaps in addition, several groups have reported that EGFR levels decrease during pregnancy and lactation [(84,85) and K.M. Darcy et al., manuscript in preparation], and we have recently determined that EGFR levels decline in conjunction with the morphological

and functional differentiation of MEC in primary culture (K.M. Darcy et al., manuscript in preparation).

In any case, the different effects of TNFα and EGF on DNA synthesis and cell number at different times in culture strongly suggest that the mitogenic actions of TNFα and EGF are mediated through independent pathways, even though there may be cooperativity between these pathways under some circumstances. TNFα may also be acting on a cell population that is unresponsive to EGF. For example, both TNFα and EGF may be able to regulate the growth and differentiation of immature MEC, while TNFα may also be able to stimulate the proliferation of a putative stem cell population and/or effect the death of a small subset of cells early in culture.

The EGFR tyrosine kinase inhibitor did not block the TNFα-induced morphological development of normal MEC The extensive branching alveolar morphogenesis that was stimulated by both concentrations of TNFα was completely unaffected by PD158780, suggesting that the pathway of TNFα-induced morphological development is not dependent on EGFR tyrosine kinase activity. This conclusion is further supported by the observation that TNFα actually induced the formation of larger colonies with more expansive ductal branching than EGF (Figure 20). This difference between TNFα- and EGF-stimulated morphogenesis may be due to the differential ability of TNFα and EGF to modulate the production and/or activity of matrix metalloproteinases (MMPs) which could then affect the remodeling of the ECM. Previous studies in our laboratory have shown that MMP-9 (the 92 kDa type IV collagenase) activity in conditioned medium from MEC was increased by TNFα but decreased by EGF [(86) and unpublished observations]. Therefore, the TNFα-induced remodeling of the ECM may permit the pronounced ductal branching and alveolar morphogenesis that occurs in response to this cytokine, while the inability of EGF to increase MMP-9 activity may explain why EGF does not stimulate branching morphogenesis to the same extent as TNFα.

In addition, it should also be noted that PD158780 alone had several interesting morphological effects. Earlier studies in our laboratory have shown that in the absence of EGF, branching alveolar morphogenesis was decreased and both atypical hybrid and squamous colony formation was enhanced (2,37). In the current studies, however, treatment of the MEC with an inhibitor of EGFR tyrosine kinase activity actually permitted alveolar morphogenesis, although the PD158780-treated colonies were smaller than those treated with either EGF or TNFα. Since PD158780 was not present for the first 5 days of culture, endogenous EGFR ligands produced by the MEC or within the ECM may have initiated the process of morphological differentiation such that the subsequent inhibition of EGFR tyrosine kinase activity was unable to inhibit morphogenesis once it had begun. This hypothesis concurs with previous studies in our laboratory in which alveolar morphogenesis proceeded even when EGF was removed from the culture medium after the first few days of culture (37). However, PD158780 also induced the apparent disintegration of the alveolar colonies when added alone or in combination with EGF (but not in the presence of TNFα), so even though PD158780 did not inhibit alveolar morphogenesis, inhibition of the EGFR tyrosine kinase activity resulted in the apoptotic death of the lobulo-alveolar organoids (87). When taken together with the aforementioned data on the effects of EGF on DNA synthesis at different times, as well as with earlier studies by both our laboratory (37) and Nancy Hynes group (88), it thus appears that EGF is needed at early times in culture (e.g. day 7) for proliferation as well as for rendering MEC competent to respond to lactogenic hormones. After differentiation, however, EGF may become a survival factor which serves to prevent apoptosis and maintain the existing state of the gland rather than stimulate further growth and/or development.

The EGFR tyrosine kinase inhibitor did not block the effects of TNFα on MEC functional differentiation In accordance with our previous studies, casein accumulation by MEC was inhibited by a 16 day treatment with 40 ng/ml TNFα. Since PD158780 was unable to alter this effect, it appears that the EGFR is not involved in the pathway whereby 40 ng/ml TNFα regulates casein accumulation. In contrast to our previous studies, casein accumulation was not increased after long-term treatment with 2 ng/ml TNFα. This apparent discrepancy may be due to the fact that TNFα was not added until day 5 in the current study, while TNFα had been present from days 0-21 of culture in the former studies. In addition, the stimulatory effect of 2 ng/ml TNFα on casein may have been masked because casein levels in the control group were unusually high in this series of experiments; this phenomenon may be due to the activity of endogenous EGFR ligands either produced by the MEC themselves or present in the RBM matrix.

Surprisingly, casein accumulation was *increased* in MEC which had been treated for 16 days with PD158780 alone or in combination with EGF. Since studies by our laboratory have shown that casein accumulation by MEC is significantly decreased in the absence of EGF, this observation was somewhat unexpected. It could be argued that this PD158780-induced increase in casein may be due, in part, to the 2-fold increase in the percentage of the alveolar, casein-producing colonies in this group; however, several factors argue against this explanation. First, in both EGF and EGF plus PD158780 treated cultures, the percentage of alveolar colonies was increased to the same extent compared to the control group, yet casein levels were higher in MEC treated with both EGF and PD158780 than in MEC treated with EGF alone. In addition, TNFα also increased the percentage of alveolar colonies, but casein accumulation by TNFα-treated MEC was either decreased or unaffected, depending on the TNFα concentration. Therefore, the increased percentage of alveolar colonies cannot completely account for the increase in casein levels in the PD158780-treated cultures.

Potential role of TNFα during mammary carcinogenesis

Lastly, a preliminary investigation suggests that both TNFα and TNF receptor expression are decreased in DMBA- and NMU-induced rat mammary carcinomas when compared to normal MEC. Given that TNFα is either cytotoxic or cytostatic to many breast cancer cells, the decrease in TNF receptor expression was not expected; however, the overall significance of these findings is, as yet, unclear. Since previous studies by our laboratory as well as experiments reported herein indicate that TNFα stimulates the proliferation and morphogenesis and regulates the function of normal MEC, it is crucial to determine when the cellular response of MEC to TNFα changes during mammary carcinogenesis. In other words, when does MEC proliferation in response to TNFα cease and cell death or stasis begin? In agreement with published reports demonstrating that TNFα can stimulate the proliferation of hyperplastic alveolar nodule (HAN) cells and enhance the progression of HAN to tumor *in vivo* (89), TNFα was found to stimulate the proliferation of both NMU-initiated and -transformed MEC organoids in primary culture. The small proliferative response of transformed MEC in primary culture was in direct contrast to the growth inhibitory or cytotoxic effect of TNFα on breast cancer cell lines in culture. Whether this alteration in response reflects the difference between primary cells in culture versus established cell lines, the presence of an extracellular matrix in the primary culture system, or both remains to be determined. In addition, TNFα significantly affected the morphological development of both the initiated and transformed MEC, inducing the formation

of lobulo-alveolar organoids from atypical colonies. At this time it is unclear whether this effect is due to the ability of TNFα to kill off a particular tumor cell population while fostering the outgrowth of another, or whether TNFα is actually able to alter the developmental path of the existing cells; however, both the data on viable cell number during the first week of culture (Figures 27 and 28) as well as preliminary histological evidence (data not shown) fail to show any evidence of significant cell death. Thus, this intriguing phenomenon requires further investigation.

Finally, the particular factors involved in the alteration in response of MEC to TNFα during mammary carcinogenesis must also be determined. For example, there may be changes in the signaling pathways triggered by the TNF receptors, such as alterations in the expression and/or function of the TNF receptor-associated signaling molecules, the TRAPs (p55) and TRAFs (p75). Specifically, a novel member of the TRAF protein family, designated CART-1, has been shown to be expressed exclusively in epithelial breast carcinoma cells, so it is possible that the different effects of TNFα in normal and malignant breast cells may be due to alterations in the expression of receptor associated signal transduction molecules; there may also be other, as yet unidentified proteins whose expression or function changes in mammary tumors. Lastly, the expression of downstream proteins required for signaling TNFα-induced cell death may be different in normal and cancerous breast tissue. For example, Bargou and co-workers (90) have recently shown that the expression of bcl-2 and bcl-x_L does not differ markedly in normal breast epithelium and cancer; however, bax-α, which promotes apoptosis, is expressed in normal breast tissue but not in cancerous breast epithelium. In addition, recent evidence has suggested that the activation of NFκB by TNFα may turn on genes which can protect against TNFα-induced cell death (91-93), and Sonenshein's group at Boston University has data that indicates that NFκB is constitutively activated in human breast cancer cells but not in normal breast cells (94). Thus, in the absence of bax-α or the presence of constitutively-activated NFκB, TNFα may be unable to elicit its cytotoxic effects or may only be able to induce the cytostasis rather than the death of breast cancer cells.

SUMMARY AND CONCLUSIONS

In conclusion, these studies have demonstrated for the first time that normal MEC produce TNFα and express mRNA for both the p55 and p75 TNF receptors. Furthermore, the levels of all three were found to be specifically and independently regulated during the various stages of mammary gland development. TNFα mRNA increased significantly during pregnancy and then decreased throughout lactation and involution, while the 26-kDa transmembrane form of TNFα protein increased during pregnancy and lactation and disappeared during involution. In contrast, p55 TNF receptor mRNA levels peaked in early lactation and declined thereafter, whereas p75 TNF receptor mRNA levels rose steadily through lactation and remained elevated during involution. TNFα was also shown to stimulate the growth of MEC *in vitro* through the p55 receptor and signaling via either TNF receptor was able to stimulate the morphological development of MEC organoids in culture. In addition, the two TNF receptors were found to have opposing effects on casein milk protein accumulation, with inhibition being signaled via p55 and stimulation via p75. Although the exact functions of TNFα in the mammary gland are still speculative, these data strongly suggest that TNFα is a physiological regulator of several key processes during mammary gland development. Because both TNFα and p55 TNF receptor mRNA expression were markedly increased during pregnancy and since p55 was found to be the sole mediator of the TNFα-induced proliferation of MEC in culture, it is believed that during pregnancy, the TNFα-induced stimulation of MEC growth and morphogenesis is signaled primarily via the p55 receptor. Since signaling through the p55 receptor was also shown to decrease casein protein synthesis and accumulation by MEC in culture, TNFα may also act via p55 to inhibit casein producion until the onset of lactation. Furthermore, the expression of both p75 TNF receptor mRNA and the transmembrane form of TNFα were increased during lactation, and functional studies of the TNF receptors showed that signaling via p75 actually stimulated casein accumulation by MEC in culture. When considered in conjunction with a previous report suggesting that the membrane forms of TNFα acts primarily via p75 (61), this data suggests that transmembrane TNFα acts via the p75 receptor to stimulate casein production during lactation.

Additional studies were then undertaken in order to test the hypothesis that the post-receptor pathway whereby TNFα modulates MEC growth and differentiation may be indirect via the up-regulation of EGFR ligand expression and/or subsequent activation of the EGFR. To evaluate this possibility, a specific inhibitor of the EGFR tyrosine kinase activity, PD 158780, was used in an attempt to inhibit the effects of TNFα on MEC in primary culture. In contrast to our original expectations, these studies demonstrated that EGFR activation is not necessary for TNFα to modulate MEC morphogenesis or functional differentiation; however, the stimulatory effect of TNFα on DNA synthesis in MEC appears to have both EGFR-dependent and -independent components. Specifically, the data suggests that the post-receptor signaling pathways whereby TNFα induces proliferation of normal MEC may differ at varying TNFα concentrations, with low concentrations of TNFα requiring a cooperativity of TNF receptor signaling with the EGFR and higher concentrations of TNFα acting independently of the EGFR. Furthermore, these studies also indicate that the cellular response to signaling from the EGFR changes during normal MEC development in primary culture. Given the apparent change in the expression and/or function of the EGFR during MEC development in vitro, further analysis of both the mechanism of TNFα action and the role of EGF are required in order to determine their respective contributions to the regulation of mammary gland development.

Lastly, in DMBA- and NMU-induced mammary tumors, both TNFα and p55 TNF receptor mRNA levels were found to be decreased in comparison to normal MEC, while p75 TNF receptor mRNA expression was undetectable. In addition, TNFα was found to stimulate the proliferation of both NMU-initiated and, albeit to a much lesser extent, transformed MEC, and significantly affected the morphological development of tumor-derived MEC. The significance of the changes in both TNFα and TNF receptor mRNA in mammary carcinomas remains to be determined, and the underlying factors responsible for the response of the tumor-derived MEC to TNFα requires further investigation.

REFERENCES

1. Marshall E 1993 Search for a Killer: Focus Shifts From Fat to Hormones. Science 259:618-621

2. Ip M M, Shoemaker S F, Darcy K M 1992 Regulation of rat mammary epithelial cell proliferation and differentiation by tumor necrosis factor α. Endocrinol 130:2833-2844

3. Urban J L, Shepard H M, Rothstein J L, Sugarman B J, Schreiber H 1986 Tumor necrosis factor: A potent effector molecule for tumor cell killing by activated macrophages. Proc Natl Acad Sci U S A 83:5233-5237

4. Larrick J W, Wright S C 1990 Cytotoxic mechanism of tumor necrosis factor-α. FASEB J 4:3215-3223

5. Sugarman B J, Aggarwal B B, Hass P E, Figari I S, Palladino M A 1985 Recombinant human tumor necrosis factor-α: Effects on proliferation of normal and transformed cells *in vitro*. Science 230:943-945

6. Takeda K, Iwamoto S, Sugimoto H, Takuma T, Kawatani N, Noda M, Masaki A, Morise H, Arimura H, Konno K 1986 Identity of differentiation inducing factor and tumour necrosis factor. Nature 323:338-340

7. Perez C, Albert I, DeFay K, Zachariades N, Gooding L, Kriegler M 1990 A nonsecretable cell surface mutant of tumor necrosis factor (TNF) kills by cell-to-cell contact. Cell 63:251-258

8. Kriegler M, Perez C, DeFay K, Albert I, Lu S D 1988 A novel form of TNF/Cachectin is a cell surface cytotoxic transmembrane protein: Ramifications for the complex physiology of TNF. Cell 53:45-53

9. Han J, Thompson P, Beutler B 1990 Dexamethasone and pentoxifylline inhibit endotoxin-induced cachectin/tumor necrosis factor synthesis at separate points in the signalling pathway. J Exp Med 172:391-394

10. Morrison D C, Ryan J L 1987 Endotoxins and disease mechanisms. Ann Rev Med 38:417-432

11. Kitabayashi A, Hirokawa M, Hatano Y, Lee M, Kuroki J, Niitsu H, Miura A B 1995 Granulocyte colony-stimulating factor downregulates allogeneic immune responses by posttranscriptional inhibition of tumor necrosis factor-α production. Blood 86:2220-2227

12. Hohmann H-P, Remy R, Brockhaus M, van Loon A P G M 1989 Two different cell types have different major receptors for human tumor necrosis factor (TNF alpha). J Biol Chem 264:14927-14934

13. Engelmann H, Holtmann H, Brakebusch C, Avni Y S, Sarov I, Nophar Y, Hadas E, Leitner O, Wallach D 1990 Antibodies to a soluble form of a tumor necrosis factor (TNF) receptor have TNF-like activity. J Biol Chem 265:14497-14504

14. Brockhaus M, Schoenfeld H J, Schlaeger E J, Hunziker W, Lesslauer W, Loetscher H 1990 Identification of two types of tumor necrosis factor receptors on human cell lines by monoclonal antibodies. Proc Natl Acad Sci U S A 87:3127-3131

15. Tartaglia L A, Weber R F, Figari I S, Reynolds C, Palladino M A, Goeddel D V 1991 The two different receptors for tumor necrosis factor mediate distinct cellular responses. Proc Natl Acad Sci U S A 88:9292-9296

16. Tartaglia L A, Rothe M, Hu Y-F, Goeddel D V 1993 Tumor necrosis factor's cytotoxic activity is signaled by the p55 TNF receptor. Cell 73:213-216

17. Wong G H W, Tartaglia L A, Lee M S, Goeddel D V 1992 Antiviral activity of tumor necrosis factor (TNF) is signaled through the 55-kDa receptor, type I TNF. J Immunol 149:3350-3353

18. Vilcek J, Palombella V J, Henriksen-DeStefano D, Swenson C, Feinman R, Hirai M, Tsujimoto M 1986 Fibroblast growth enhancing activity of tumor necrosis factor and its relationship to other polypeptide growth factors. J Exp Med 163:632-643

19. Kalthoff H, Roeder C, Brockhaus M, Thiele H, Schmiegel W 1993 Tumor necrosis factor (TNF) up-regulates the expression of p75 but not p55 TNF receptors, and both receptors mediate, independently of each other, up-regulation of transforming growth factor α and epidermal growth factor receptor mRNA. J Biol Chem 268:2762-2766

20. Jacobsen F W, Rothe M, Rusten L, Goeddel D V, Smeland E B, Veiby O P, Slordal L, Jacobsen S E W 1994 Role of the 75-kDa tumor necrosis factor receptor: Inhibition of early hematopoiesis. Proc Natl Acad Sci U S A 91:10695-10699

21. Vandenabeele P, Declercq W, Vercammen D, Van de Craen M, Grooten J, Loetscher H, Brockhaus M, Lesslauer W, Fiers W 1992 Functional characterization of the human tumor necrosis factor receptor p75 in a transfected rat/mouse T cell hybridoma. J Exp Med 176:1015-1024

22. Grell M, Zimmermann G, Hülser D, Pfizenmaier K, Scheurich P 1994 TNF receptors TR60 and TR80 can mediate apoptosis via induction of distinct signal pathways. J Immunol 153:1963-1972

23. Lægreid A, Medvedev A, Nonstad U, Bombara M P, Ranges G, Sundan A, Espevik T 1994 Tumor necrosis factor receptor p75 mediates cell-specific activation of nuclear factor kappaB and induction of human cytomegalovirus enhancer. J Biol Chem 269:7785-7791

24. Dollbaum C, Creasey A A, Dairkee S H, Hiller A J, Rudolph A R, Lin L, Vitt C, Smith H S 1988 Specificity of tumor necrosis factor toxicity for human mammary carcinomas relative to normal mammary epithelium and correlation with response to doxorubicin. Proc Natl Acad Sci U S A 85:4740-4744

25. Pusztai L, Lewis C E, McGee J O 1993 Growth arrest of the breast cancer cell line, T47D, by TNFα; cell cycle specificity and signal transduction. Br J Cancer 67:290-296

26. Hahm H A, Ip M M 1990 Primary culture of normal rat mammary epithelial cells within a basement membrane matrix. I. Regulation of proliferation by hormones and growth factors. In Vitro Cell Dev Biol 26:791-802

27. Hahm H A, Ip M M, Darcy K, Black J D, Shea W K, Forczek S, Yoshimura M, Oka T 1990 Primary culture of normal rat mammary epithelial cells within a basement membrane matrix. II. Functional differentiation under serum-free conditions. In Vitro Cell Dev Biol 26:803-814

28. Darcy K M, Black J D, Hahm H A, Ip M M 1991 Mammary organoids from immature virgin rats undergo ductal and alveolar morphogenesis when grown within a reconstituted basement membrane. Exp Cell Res 196:49-65

29. Shirai T, Shimizu N, Horiguchi S, Ito H 1989 Cloning and expression in *Escherichia coli* of the gene for rat tumor necrosis factor. Agr Biol Chem 53:1733-1736

30. Lewis M, Tartaglia L A, Lee A, Bennett G L, Rice G C, Wong G H W, Chen E Y, Goeddel D V 1991 Cloning and expression of cDNAs for two distinct murine tumor necrosis factor receptors demonstrate one receptor is species specific. Proc Natl Acad Sci U S A 88:2830-2834

31. Danielson P E, Forss-Petter S, Brow M A, Calavetta L, Douglass J, Milner R J, Sutcliffe J G 1988 p1b15: a cDNA clone of the rat mRNA encoding cyclophilin. DNA 7:261-267

32. Darcy K M 1993 Hormone regulation of normal rat mammary epithelial cell growth, branching morphogenesis, and functional differentiation in primary culture. 1-294 (Abstract)

33. Church G M, Gilbert W 1984 Genomic sequencing. Proc Natl Acad Sci U S A 81:1991-1995

34. De M, Sanford T H, Wood G W 1992 Detection of interleukin-1, interleukin-6, and tumor necrosis factor-α in the uterus during the second half of pregnancy in the mouse. Endocrinol 131:14-20

35. Laemmli U K 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685

36. Bradford M M 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248-254

37. Darcy K M, Shoemaker S F, Lee P-P H, Vaughan M M, Black J D, Ip M M 1995 Prolactin and epidermal growth factor regulation of the proliferation, morphogenesis, and functional differentiation of normal rat mammary epithelial cells in three dimensional primary culture. J Cell Physiol 163:346-364

38. Ignatowski T A, Spengler R N 1994 Tumor necrosis factor-α: presynaptic sensitivity is modified after antidepressant drug administration. Brain Res 665:293-299

39. Welsch C W 1985 Host factors affecting the growth of carcinogen-induced rat mammary carcinomas: a review and tribute to Charles Brenton Huggins. Cancer Res 45:3415-3443

40. Thompson H J, Adlakha H 1991 Dose-responsive induction of mammary gland carcinomas by the intraperitoneal injection of 1-methyl-1-nitrosourea. Cancer Res 51:3411-3415

41. De S K, Chen H-L, Pace J L, Hunt J S, Terranova P F, Enders G C 1993 Expression of tumor necrosis factor-α in mouse spermatogenic cells. Endocrinol 133:389-396

42. Marquis S T, Rajan J V, Wynshaw-Boris A, Xu J, Yin G-Y, Abel K J, Weber B L, Chodosh L A 1995 The developmental pattern of *Brca 1* expression implies a role in differentiation of the breast and other tissues. Nature Genetics 11:17-26

43. Pocsik E, Duda E, Wallach D 1995 Phosphorylation of the 26 kDa TNF precursor in monocytic cells and in transfected HeLa cells. J Inf 45:152-160

44. Pandita R, Pocsik E, Aggarwal B B 1992 Interferon-gamma induces cell surface expression for both types of tumor necrosis factor receptors. FEBS Lett 312:87-90

45. Sheehan K C F, Pinckard J K, Arthur C D, Dehner L P, Goeddel D V, Schreiber R D 1995 Monoclonal antibodies specific for murine p55 and p75 tumor necrosis factor receptors: Identification of a novel in vivo role for p75. J Exp Med 181:607-617

46. Grazioli L, Casero D, Restivo A, Cozzi E, Marcucci F 1994 Tumor necrosis factor-driven formation of disulfide-linked receptor aggregates. J Biol Chem 269:22304-22309

47. Shalaby M, Sundan A, Loetscher H, Brockhaus M, Lesslauer W, Espevik T 1990 Binding and regulation of cellular functions by monoclonal antibodies against human tumor necrosis factor receptors. J Exp Med 172:1517-1520

48. De Kossodo S, Cruz P D, Dougherty I, Thompson P, Silva-Valdez M, Beutler B 1995 Expression of the tumor necrosis factor gene by dermal fibroblasts in response to ultraviolet irradiation or lipopolysaccharide. J Invest Dermatol 104:318-322

49. Beil W J, Weller P F, Peppercorn M A, Galli S J, Dvorak A M 1995 Ultrastructural immunogold localization of subcellular sites of TNF-α in colonic Crohn's disease. J Leukocyte Biol 58:284-298

50. Havell E A, Rogerson B J 1993 Endotoxin-induced tumor necrosis factor alpha synthesis in murine embryo fibroblasts. Infect Immun 61:1630-1635

51. Pusztai L, Clover L M, Cooper K, Starkey P M, Lewis C E, McGee J O 1994 Expression of tumour necrosis factor α and its receptors in carcinoma of the breast. Br J Cancer 70:289-292

52. Miles D W, Happerfield L C, Naylor M S, Bobrow L G, Rubens R D, Balkwill F R 1994 Expression of tumour necrosis factor (TNFα) and its receptors in benign and malignant breast tissue. Int J Cancer 56:777-782

53. Schmiegel W, Roeder C, Schmielau J, Rodeck U, Kalthoff H 1993 Tumor necrosis factor α induces the expression of transforming growth factor α and the epidermal growth factor receptor in human pancreatic cancer cells. Proc Natl Acad Sci U S A 90:863-867

54. Woodworth C D, McMullin E, Iglesias M, Plowman G D 1995 Interleukin 1α and tumor necrosis factor α stimulate autocrine amphiregulin expression and proliferation of human papillomavirus-immortalized and carcinoma-derived cervical epithelial cells. Proc Natl Acad Sci U S A 92:2840-2844

55. Rewcastle G W, Palmer B D, Thompson A M, Bridges A J, Cody D R, Zhou H R, Fry D W, McMichael A, Denny W A 1996 Tyrosine kinase inhibitors. 10. Isomeric 4-[(3-bromophenyl)amino]pyrido[d]- pyrimidines are potent ATP-binding site inhibitors of the tyrosine kinase function of the epidermal growth factor receptor. J Med Chem 39:1823-1835

56. Basolo F, Conaldi P G, Fiore L, Calvo S, Toniolo A 1993 Normal breast epithelial cells produce interleukins 6 and 8 together with tumor-necrosis factor: Defective IL6 expression in mammary carcinoma. Int J Cancer 55:926-930

57. Yelavarthi K K, Hunt J S 1993 Analysis of p60 and p80 tumor necrosis factor-α receptor messenger RNA and protein in human placentas. Am J Pathol 143:1131-1141

58. Hobbs A A, Richards D A, Kessler D J, Rosen J M 1982 Complex hormonal regulation of rat casein gene expression. J Biol Chem 257:3598-3605

59. Thompson A M, Bridges A J, Fry D W, Kraker A J, Denny W A 1995 Tyrosine kinase inhibitors. 7. 7-amino-4-(phenylamino)- and 7-amino-4-[(phenylmethyl)amino]pyrido[4,3-d]pyrimidines: A new class of inhibitors of the tyrosine kinase activity of the epidermal growth factor receptor. J Med Chem 38:3780-3788

60. Traurig H H 1967 Cell proliferation in the mammary gland during late pregnancy and lactation. Anat Rec 157:489-504

61. Grell M, Douni E, Wajant H, Löhden M, Clauss M, Maxeiner B, Georgopoulos S, Lesslauer W, Kollias G, Pfizenmaier K, Scheurich P 1995 The transmembrane form of tumor necrosis factor is the prime activating ligand of the 80 kDa tumor necrosis factor receptor. Cell 83:793-802

62. Bradley J R, Thiru S, Pober J S 1995 Disparate localization of 55-kd and 75-kd tumor necrosis factor receptors in human endothelial cells. Am J Pathol 146:27-32

63. Laster S M, Wood J G, Gooding L R 1988 Tumor necrosis factor can induce both apoptic and necrotic forms of cell lysis. J Immunol 141:2629-2634

64. Tartaglia L A, Ayres T M, Wong G H W, Goeddel D V 1993 A novel domain within the 55 kd TNF receptor signals cell death. Cell 74:845-853

65. Espevik T, Brockhaus M, Loetscher H, Nonstad U, Shalaby R 1990 Characterization of binding and biological effects of monoclonal antibodies against a human tumor necrosis factor receptor. J Exp Med 171:415-426

66. Pagliacci M C, Fumi G, Migliorati G, Grignani F, Riccardi C, Nicoletti I 1993 Cytostatic and cytotoxic effects of tumor necrosis factor α on MCF-7 human breast tumor cells are differently inhibited by glucocorticoid hormones. Lymphokine and Cytokine Research 12:439-447

67. Patton J S, Shepard H M, Wilking H, Lewis G, Aggarwal B B, Eessalu T E, Gavin L A, Grunfeld C 1986 Interferons and tumor necrosis factors have similar catabolic effects on 3T3 L1 cells. Proc Natl Acad Sci U S A 83:8313-8317

68. Kern P A 1988 Recombinant human tumor necrosis factor does not inhibit lipoprotein lipase in primary culture of isolated human adipocytes. J Lipid Res 29:909-914

69. Martel-Pelletier J, Mineau F, Jolicoeur F C, Pelletier J P 1995 Molulation of TNFSR55 and TNFSR75 by cytokines and growth factors in human synovial fibroblasts. J Rheum (Supp) 43:115-119

70. Butler D M, Feldmann M, Di Padova F, Brennan F M 1994 p55 and p75 tumor necrosis factor receptors are expressed and mediate common functions in synovial fibroblasts and other fibroblasts. Eur Cytokine Netw 5:441-448

71. Feingold K R, Doerrler W, Dinarello C A, Fiers W, Grunfeld C 1992 Stimulation of lipolysis in cultured fat cells by tumor necrosis factor, interleukin-1, and the interferons is blocked by inhibition of prostaglandin synthesis. Endocrinol 130:10-16

72. Elias J J, Pitelka D R, Armstrong R C 1973 Changes in fat cell morphology during lactation in the mouse. Anat Rec 177:533-547

73. Feingold K R, Grunfeld C 1987 Tumor necrosis factor-alpha stimulates hepatic lipogenesis in the rat *in vivo*. J Clin Invest 80:184-190

74. Westwick J K, Weitzel C, Minden A, Karin M, Brenner D A 1994 Tumor necrosis factor α stimulates AP-1 activity through prolonged activation of the c-Jun kinase. J Biol Chem 269:26396-26401

75. Gardner A M, Johnson G L 1996 Fibroblast growth factor-2 suppression of tumor necrosis factor α-mediated apoptosis requires Ras and the activation of mitogen-activated protein kinase. J Biol Chem 271:14560-14566

76. Westwick J K, Bielawska A E, Dbaibo G, Hannun Y A, Brenner D A 1995 Ceramide activates the stress-activated protein kinases. J Biol Chem 270:22689-22692

77. Vietor I, Schwenger P, Li W, Schlessinger J, Vilcek J 1993 Tumor necrosis factor-induced activation and increased tyrosine phosphorylation of mitogen-activated protein (MAP) kinase in human fibroblasts. J Biol Chem 268:18994-18999

78. Winston B W, Riches D W H 1995 Activation of $p42^{mapk/erk2}$ following engagement of tumor necrosis factor receptor CD120a (p55) in mouse macrophages. J Immunol 155:1525-1533

79. Winston B W, Remigio L K, Riches D W H 1995 Preferential involvement of MEK1 in the tumor necrosis factor-α-induced activation of $p42^{mapk/erk2}$ in mouse macrophages. J Biol Chem 270:27391-27394

80. Yao B, Zhang Y, Delikat S, Mathias S, Basu S, Kolesnick R 1995 Phosphorylation of Raf by ceramide-activated protein kinase. Nature 378:307-310

81. Sluss H K, Barrett T, Derijard B, Davis R J 1994 Signal transduction by tumor necrosis factor mediated by JNK protein kinases. Mol Cell Biol 14:8376-8384

82. Palombella V J, Vilcek J 1989 Mitogenic and cytotoxic actions of tumor necrosis factor in BALB/c 3T3 cells. J Biol Chem 264:18128-18136

83. Lewis G D, Aggarwal B B, Eessalu T E, Sugarman B J, Shepard H M 1987 Modulation of the growth of transformed cells by human tumor necrosis factor-α and interferon-gamma. Cancer Res 47:5382-5385

84. Edery M, Pang K, Larson L, Colosi T, Nandi S 1985 Epidermal growth factor receptor levels in mouse mammary glands in various physiological states. Endocrinol 117:405-411

85. Moorby C D, Taylor J A, Forsyth I A 1995 Transforming growth factor-α: Receptor binding and action on DNA synthesis in the sheep mammary gland. J Endocrinol 144:165-171

86. Lee P-P, Hwang J J, Ip M M 1997 Functional significance of matrix metalloproteinases (MMP) in mammary epithelial cells (MEC). Proc Am Cancer Res 38:406(Abstract)

87. Darcy K M, Wohlhueter A, Shoemaker S, Lee P-P, Varela L M, Ip M M 1997 Differential effects of epidermal growth factor receptor (EGFR) inhibition in mammary epithelial cells (MEC). Proc Am Cancer Res 38:56(Abstract)

88. Merlo G R, Graus-Porta D, Cella N, Marte B M, Taverna D, Hynes N E 1996 Growth, differentiation and survival of HC11 mammary epithelial cells: Diverse effects of receptor tyrosine kinase-activating peptide growth factors. Eur J Cell Biol 70:97-105

89. Tsai S-C, Miller B E, Jones R F, Heppner G H 1997 Tumor necrosis factor α-induced stimulation of neoplastic progression of preneoplastic, hyperplastic alveolar nodule line C4. Int J Oncol 10:1209-1215

90. Bargou R C, Wagener C, Bommert K, Mapara M Y, Daniel P T, Arnold W, Dietel M, Guski H, Feller A, Royer H D, Dorken B 1996 Overexpression of the death-promoting gene bax-alpha which Is downregulated in breast cancer restores sensitivity to different apoptotic stimuli and reduces tumor growth in SCID mice. J Clin Invest 97:2651-2659

91. Beg A A, Baltimore D 1996 An essential role for NF-kB in preventing TNF-α-induced cell death. Science 274:782-784

92. Wang C-Y, Mayo M W, Baldwin A S 1996 TNF- and cancer therapy-induced apoptosis: potentiation by inhibition of NF-kB. Science 274:784-787

93. Van Antwerp D J, Martin S J, Kafri T, Green D R, Verma I M 1996 Suppression of TNF-α-induced apoptosis by NF-kB. Science 274:787-789

94. Barinaga M 1996 Life-death balance within the cell. Science 274:724

APPENDICES

I. FIGURES

see attached pages

II. FIGURE LEGENDS

Figure Legends

Figure 1. Morphological appearance of freshly isolated mammary epithelial organoids. Epithelial cell organoids were isolated from the mammary glands of virgin, pregnant, lactating and post-lactational rats using mechanical and enzymatic digestion followed by differential filtration. A, puberty, age 50 days; B, day 14 pregnancy; C, day 5 lactation; D, day 10 lactation; E, day 15 lactation; F, day 21 lactation, 12 hour post-weaning; G, one week post-lactation (involution). Magnification in all pictures is the same (28.9X).

Figure 2. Western blot of casein protein in freshly isolated mammary epithelial organoids. Equivalent amounts of protein (2.5 μg) were loaded into each lane. Lane 1, puberty, age 50 days; lane 2, day 14 pregnancy; lane 3, day 5 lactation; lane 4, day 10 lactation; lane 5, day 15 lactation; lane 6, day 21 lactation, 12 hour post-weaning; lane 7, one week post-lactation.

Figure 3. Expression of TNFα mRNA in normal MEC. A, Northern blot of TNFα mRNA. Approximately 1.0 - 1.5 μg of poly A^+ mRNA from freshly isolated MEC was loaded into each lane, and 2 x 10^6 cpm/ml of ^{32}P-labeled TNFα cDNA probe was used for hybridization. The blot was exposed to either a PhosphorImager screen or x-ray film for 48 hours. As a positive control, total RNA from lipopolysaccharide-stimulated J774A.1 macrophage cells was also probed and shown to express a single transcript of 1.9 kb (data not shown). This Northern blot is representative of 4 independent experiments. B, Normalization of TNFα to GAPDH (represents the experiment shown in A). C, Normalization of TNFα to p1B15 (cyclophilin) (represents the experiment shown in A).

Figure 4. Expression of TNFα protein in normal MEC. This figure shows a representative (of 4 independent experiments) Western blot of TNFα protein. Equivalent amounts of protein (50 μg/lane) from freshly isolated MEC were loaded into each lane. Lane 1, puberty, age 50 days; lane 2, day 14 pregnancy; lane 3, day 5 lactation; lane 4, day 10 lactation; lane 5, day 15 lactation; lane 6, day 21 lactation, 12 hour post-weaning; lane 7, one week post-lactation (involution). Purified, soluble mouse TNFα was used as a positive control and was found to be 17-kDa (data not shown). The upper arrow indicates 26-kDa transmembrane TNFα, and the lower arrow indicates 17-kDa soluble TNFα.

Figure 5. Northern blot analysis of p55 TNF receptor mRNA from normal MEC. A, Northern blot of p55 TNF receptor mRNA. Approximately 1.0 - 1.5 μg of poly A^+ mRNA from freshly isolated MEC were loaded into each lane, and 2 x 10^6 cpm/ml of ^{32}P-labeled p55 TNF receptor cDNA probe was used for hybridization. The blot was exposed to either a PhosphorImager

screen or film for 48 hours. As a positive control, total RNA from Wehi-164 cells was also probed and shown to express a single transcript of 2.3 kb (data not shown). This Northern blot is representative of four independent experiments. B, Normalization of p55 mRNA levels to GAPDH (represents the experiment shown in A).

Figure 6. Northern blot analysis of p75 TNF receptor mRNA from normal MEC. A, Northern blot of p75 TNF receptor mRNA. Approximately 1.0 - 1.5 μg of poly A^+ mRNA from freshly isolated MEC were loaded into each lane, and 2 x 10^6 cpm/ml of ^{32}P-labeled p75 TNF receptor cDNA probe was used for hybridization. The blot was exposed to either a PhosphorImager screen or film for 7 days. As a positive control, total RNA from Wehi-164 cells was also probed and shown to express a 3.6 kb transcript as previously reported {4000} (data not shown). This Northern blot is representative of four independent experiments. B, Normalization of p75 mRNA levels to GAPDH (represents the experiment shown in A).

Figure 7. Western blot analysis of p55 TNF receptor protein expression on MEC during lactation. Five microliters (5 μl) of each of the anti-p55 TNF receptor immunoprecipitates (using anti-p55 antibody 55R-593.4.48 at 10 μg/ml) from days 5 and 10 of lactation were loaded into their respective lanes (lanes 1 and 2), and 30 μl of a rat spleen membrane preparation was loaded into lane 3. The Western blot on the left was incubated with anti-p55 TNF receptor antibody 55R-593.4.48 (2 μg/ml), while the Western blot on the right was incubated with normal Armenian hamster serum (2 μg/ml, prepared in house). Both sera identified bands at approximately 55-60 kDa, the expected molecular weight for the p55 TNF receptor.

Figure 8. Northern blot analysis of TNFα mRNA expression in normal MEC and fibroblasts during puberty. Approximately 1.0 - 1.5 μg of poly A^+ mRNA from freshly isolated MEC or cultured fibroblasts was loaded into each lane, and 2 x 10^6 cpm/ml of ^{32}P-labeled TNFα cDNA probe was used for hybridization. The blot was exposed to either a PhosphorImager screen or x-ray film for 48 hours. As a positive control, total RNA from lipopolysaccharide-stimulated J774A.1 macrophage cells was also probed and shown to express a single transcript of 1.9 kb (data not shown). It should be noted that both samples were analyzed on the same Northern blot, although the sample lanes were not directly adjacent. This Northern blot represents a single experiment. MEC, mammary epithelial cells; F, fibroblasts.

Figure 9. Northern blot analysis of p55 TNF receptor mRNA expression in normal MEC and fibroblasts during puberty. Approximately 1.0 - 1.5 μg of poly A^+ mRNA from either freshly isolated MEC or cultured fibroblasts was loaded into each lane, and 2 x 10^6 cpm/ml of ^{32}P-labeled p55 TNF receptor cDNA probe was used for hybridization. The blot was exposed to either a PhosphorImager screen or x-ray film for 48 hours. As a positive control, total RNA from Wehi-164 cells was also probed and shown to express a single transcript of 2.3 kb (data not shown). It should be noted that both samples were analyzed on the same Northern blot, although the sample lanes were not directly adjacent. This Northern blot represents a single experiment. MEC, mammary epithelial cells; F, fibroblasts.

Figure 10. Northern blot analysis of p75 TNF receptor mRNA expression in normal MEC and fibroblasts during puberty. Approximately 1.0 - 1.5 μg of poly A^+ mRNA from either freshly isolated MEC or cultured fibroblasts was loaded into each lane, and 2 x 10^6 cpm/ml of ^{32}P-labeled p75 TNF receptor cDNA probe was used for hybridization. The blot was exposed to either a PhosphorImager screen or x-ray film for 7 days. As a positive control, total RNA from Wehi-164 cells was also analyzed and shown to express a 3.6 kb transcript as previously reported (data not shown). It should be noted that both samples were analyzed on the same

Northern blot, although the sample lanes were not directly adjacent. This Northern blot represents a single experiment. MEC, mammary epithelial cells; F, fibroblasts.

Figure 11. Western blot analysis of TNFα protein expression in whole mammary gland extracts. Equivalent amounts of protein (50 μg) from whole mammary gland extracts were loaded into each lane. Lane 1, puberty, age 50 days; lane 2, day 14 pregnancy; lane 3, day 5 lactation; lane 4, day 10 lactation; lane 5, day 15 lactation; lane 6, day 21 lactation, 12 hour post-weaning; lane 7, one week post-lactation. A duplicate blot incubated without primary antiserum demonstrated that the TNFα bands were specific. The upper arrow indicates the 32-kDa band, and the lower arrow indicates 26-kDa transmembrane TNFα.

Figure 12. Effect of the TNF receptor agonistic antibodies on [^{3}H]-thymidine incorporation by normal mammary epithelial cells in primary culture. MEC were cultured until day 5 in suboptimal (0.1 ng/ml EGF) medium; the medium was then changed and EGF (10 ng/ml), human TNFα (40 ng/ml) or various dilutions of the two agonistic antibodies specific for either the p55 or p75 TNF receptor were added and tested for their ability to affect [^{3}H]-thymidine incorporation by MEC after 48 hours; [^{3}H]-thymidine (5 μCi per ml of medium) was added for the last 4 hours of incubation. EGF, human TNFα, and the 1:1000 dilution of the p55 receptor antibody induced statistically significant increases in thymidine incorporation (*Significantly different from 0.1 ng/ml EGF control, $P<0.05$). Each group represents the mean ± SEM of triplicate wells. This graph is representative of 4 independent experiments.

Figure 13. Effect of EGF, human TNFα, or the TNF receptor agonistic antibodies on casein accumulation by MEC in primary culture in reduced (0.1 ng/ml) EGF medium. MEC were cultured until day 5 in suboptimal (0.1 ng/ml EGF) medium; the medium was then changed and EGF (10 ng/ml), human TNFα (40 ng/ml) or various dilutions of the two agonistic antibodies specific for either the p55 or p75 TNF receptor were added and tested for their ability to affect casein accumulation after 48 hours. A, Western blot analysis of casein protein accumulation in extracts of cells plus EHS gel. Equivalent amounts of protein (20 μg) were loaded into each lane. Each sample represents a combination of triplicate wells for each group. This Western blot is representative of two independent experiments. B, Quantitation of casein accumulation by MEC. The relative intensities of all 4 (α_1, α_2, β and γ) casein bands on each blot were quantified by densitometric analysis. Represents the experiment shown in A.

Figure 14. Effect of TNFα or TNF receptor agonistic antibodies on casein accumulation by MEC in primary culture in optimal (10 ng/ml EGF) medium. MEC were cultured until day 5 in optimal (10 ng/ml EGF) medium; the medium was then changed and human TNFα (40 ng/ml) or various dilutions of the two agonistic antibodies specific for either the p55 or p75 TNF receptor were added and tested for their ability to affect casein accumulation after 48 hours. A, Western blot analysis of casein protein accumulation in extracts of cells plus EHS gel. Equivalent amounts of protein (8 μg) were loaded into each lane. Each sample represents a combination of triplicate wells for each group. This blot is representative of two independent experiments. B, Quantitation of casein accumulation by MEC. In order to determine whether the effects of the TNF receptor agonistic antibodies were statistically significant, casein levels in each of the three individual wells per treatment group were independently analyzed. The individual triplicate samples for each group were electrophoresed on replicate gels, and the relative intensities of all 4 (α_1, α_2, β and γ) casein bands on each blot were quantified by densitometric analysis. Each bar thus represents the mean ± SEM of triplicate samples

expressed as a percentage of the control. (*Significantly different than serum-free control, $P<0.05$).

Figure 15. Effect of the TNF receptor agonistic antibodies on casein *synthesis* by MEC in primary culture. MEC were cultured until day 5 in optimal (10 ng/ml EGF) medium; the medium was then changed and various dilutions of the two agonistic antibodies specific for either the p55 or p75 TNF receptor were tested for their ability to affect casein synthesis as described in Materials and Methods. The relative intensities of all 4 (α_1, α_2, β and γ) casein bands on each blot were quantified by densitometric analysis. This figure shows that the antibodies had opposing effects on casein synthesis by MEC. The 1:1000 dilution of the p55 receptor antibody suppressed synthesis of the α_1, α_2 and γ caseins, while the p75 antibody stimulated the synthesis of all casein isoforms in a concentration-dependent manner. Each bar represents the mean ± SEM of triplicate samples. (X = not detectable; *Significantly different than control, $P<0.05$.)

Figure 16. The effects of EGF, TNFα and PD 158780 on MEC proliferation when measured on day 7 after 48 hours of treatment. MEC were cultured in EGF-free medium until day 5; the medium was then changed and the MEC were treated as indicated for 48 hours from days 5-7 of culture. The EGFR tyrosine kinase inhibitor (designated as PD 158) significantly inhibited the EGF-induced increase in thymidine incorporation when measured after 48 hours, only modestly suppressed proliferation induced by 40 ng/ml TNFα, and reduced DNA synthesis in response to 2 ng/ml TNFα by approximately 50%. (*Significantly different than 0 EGF control, $P<0.05$. +Significantly different than corresponding group without PD 158780, $P<0.05$.) Each bar represents the mean ± SEM of triplicate culture wells. This graph is representative of 3-4 experiments. Although the slight inhibitory effect of PD 158780 on thymidine incorporation in response to 40 ng/ml TNFα was statistically significant in this experiment, no significant effect was seen in three other experiments.

Figure 17. The effects of EGF, TNFα and PD 158780 on MEC proliferation when measured on day 21 of culture after 16 days of treatment. MEC were cultured in 0 EGF medium until day 5; the medium was then changed and the MEC were treated as indicated for 16 days from days 5-21 of culture. The EGFR tyrosine kinase inhibitor alone, EGF alone, or EGF in combination with PD 158780 significantly inhibited thymidine incorporation after 16 days of treatment. The proliferation of cells treated with 40 ng/ml TNFα in either the presence or absence of PD 158780 was not statistically different from the control. DNA synthesis in cells treated with 2 ng/ml TNFα was slightly reduced compared to the 0 EGF control, and PD 158780 significantly suppressed thymidine incorporation in this group. (*Significantly different than 0 EGF control, $P<0.05$. +Significantly different than corresponding group without PD 158780, $P<0.05$. #Significantly different than EGF-treated group, $P<0.05$.) Each bar represents the mean ± SEM of triplicate culture wells. This graph is representative of 2 independent experiments.

Figure 18. The effects of EGF, TNFα and PD 158780 on viable cell number when measured on day 7 after 48 hours of treatment. MEC were cultured in EGF-free medium until day 5; the medium was then changed and the MEC were treated as indicated for 48 hours from days 5-7 of culture. The EGF-induced increase in cell number was suppressed by PD 158780, while neither TNFα nor PD 158780 alone had any significant effect. (*Significantly different than 0 EGF control, $P<0.05$. +Significantly different than corresponding group without PD 158780, $P<0.05$.) Each bar represents the mean ± SEM of triplicate culture wells. This graph is representative of 3 independent experiments.

Figure 19. The effects of EGF, TNFα and PD 158780 on viable cell number when measured on day 21 of culture after 16 days of treatment. MEC were cultured in EGF-free medium until day 5; the medium was then changed and the MEC were treated as indicated for 16 days from days 5-21 of culture. PD 158780 inhibited the EGF-induced increase in cell number, but failed to significantly inhibit the increase in cell number in response to 40 ng/ml TNFα. (*Significantly different than 0 EGF control, $P<0.05$. +Significantly different than corresponding group without PD 158780, $P<0.05$. #Significantly different than EGF-treated group, $P<0.05$.) Each bar represents the mean ± SEM of triplicate culture wells. This graph is representative of 3 independent experiments.

Figure 20. The effects of EGF, TNFα and PD 158780 on the morphological appearance of MEC organoids in primary culture after 16 days of treatment. 0 EGF control: a, squamous epithelial organoids; b, atypical organoid; c, multilobular alveolar organoid. 0 EGF + PD 158: d and e, multilobular alveolar organoids; note disrupted appearance. + EGF: f and g, multilobular alveolar organoids. + EGF + PD 158: h and i, multilobular alveolar organoids; note disrupted appearance. + TNFα: j, ductal-alveolar colony network. + TNFα + PD 158: k, ductal-alveolar colony network; note the lack of effect of PD 158 in the presence of TNFα. Magnification in all photographs is the same. The magnification bars measure 100 μm.

Figure 21. Analysis of the effects of EGF, TNFα and PD 158780 on morphological differentiation of MEC organoids in primary culture. The morphologic type of each colony was classified and quantitated on day 21 of culture. Four main colony types were quantitated: end bud-like, alveolar, squamous, and atypical hybrid, and the proportion of each is expressed as a percentage of total epithelial colonies. The percentage of squamous and atypical colonies was significantly decreased in all treatment groups, and the percentage of alveolar colonies was increased when compared to the 0 EGF control. Bars represent the mean ± SEM of triplicate culture wells. (*Significantly different than 0 EGF control, $P<0.05$. +Significantly different than corresponding group without PD 158780, $P<0.05$.) This graph is representative of 3 independent experiments.

Figure 22. The effects of EGF, TNFα and PD 158780 on casein protein accumulation by MEC when measured on day 7 of culture after 48 hours of treatment. MEC were cultured in EGF-free medium until day 5; the medium was then changed and the MEC were treated as indicated for 48 hours from days 5-7. Equivalent amounts of protein (10 μg) from extracts of MEC plus RBM matrix were loaded into each lane and subjected to Western blot analysis for casein. Each lane represents a combination of triplicate wells for each group. Only EGF significantly stimulated casein accumulation at this time. This blot is representative of 3 independent experiments. A second Western blot in which loading was based on an equivalent number of cells in each lane was virtually identical.

Figure 23. The effects of EGF, TNFα, and PD 158780 on casein accumulation by MEC when measured on day 21 of culture after 16 days of treatment. MEC were cultured in EGF-free medium until day 5; the medium was then changed and the MEC were treated as indicated for 16 days from days 5-21 of culture. A, Western blot analysis of casein protein accumulation in extracts of MEC plus RBM matrix. Loading was based on an equivalent number of cells (5×10^3) per lane, and each lane represents a combination of triplicate wells for each group. This blot is representative of 3 independent experiments. B, Determination of casein accumulation by ELISA. Casein levels in extracts of MEC plus RBM matrix were analyzed by ELISA, and results are expressed as nanograms per 10^5 cells. Bars represent the mean ± SEM of triplicate

wells. (*Significantly different than 0 EGF control, $P<0.05$. +Significantly different than corresponding group without PD 158780.) The absolute micrograms of casein per well (uncorrected for cell number) were as follows: 9.44 ± 2.08 (0 EGF), 8.85 ± 1.04 (0 +PD 158), 19.6 ± 8.37 (+EGF), 16.5 ± 1.26 (+EGF +PD 158), 3.61 ± 1.43 (+TNF 40 ng/ml), 2.45 ± 0.85 (+TNF 40 ng +PD 158), 12.3 ± 1.64 (+TNF 2 ng/ml), 5.81 ± 0.79 (+TNF 2 ng +PD 158). This graph is representative of three experiments.

Figure 24. Expression of TNFα mRNA in DMBA- and NMU-induced mammary carcinomas. A, Northern blot analysis of TNFα mRNA expression. Approximately 1.0 - 1.5 μg of poly A^+ mRNA from freshly isolated, age-matched MEC or DMBA- or NMU-induced mammary tumors were probed as previously described for TNFα mRNA. The blot was exposed to either a PhosphorImager screen or x-ray film for 5 days. As a positive control, total RNA from lipopolysaccharide-stimulated J774A.1 macrophage cells was also probed and found to express a single transcript of 1.9 kb (data not shown). This Northern blot represents one experiment. B, Normalization of TNFα to GAPDH as described in the text. (represents the experiment shown in A). The level of the 1.9 kb transcript was greatly reduced in both tumor types when compared to normal MEC from virgin rats.

Figure 25. Expression of p55 TNF receptor mRNA in DMBA- and NMU-induced mammary carcinomas. A, Northern blot analysis of p55 TNF receptor mRNA expression. Approximately 1.0 - 1.5 μg of poly A^+ mRNA from freshly isolated, age-matched MEC from virgin rats or DMBA- or NMU-induced mammary tumors were probed as previously described for the p55 TNF receptor. The blot was exposed to either a PhosphorImager screen or x-ray film for 5 days. As a positive control, total RNA from Wehi-164 cells was also probed and found to express a single transcript of 2.3 kb (data not shown). This Northern blot is representative of one experiment. B, Normalization of p55 TNF receptor mRNA levels to GAPDH as described in the text. (represents the experiment shown in A). The level of the 2.3 kb p55 transcript was greatly reduced in both tumor types when compared to normal MEC from virgin rats.

Figure 26. The effects of TNFα on viable cell number of normal and NMU-initiated MEC. MEC were cultured in EGF-free medium in either the presence or absence of 4 or 40 ng/ml TNFα. The growth stimulatory effect of TNFα on both the control and NMU-initiated MEC was identical and concentration-dependent. Each data point represents the mean ± SEM of triplicate culture wells. This graph is representative of 3 independent experiments.

Figure 27. The effects of TNFα on viable cell number of normal and transformed MEC. MEC, isolated from either normal control mammary glands or from NMU-induced mammary tumors, were cultured in EGF-free medium in either the presence or absence of 40 ng/ml TNFα. While TNFα significantly stimulated the growth of the normal, control MEC, it had no significant effect on the growth of transformed MEC. Each data point represents the mean ± SEM of triplicate culture wells. This graph is representative of 2 independent experiments.

Figure 28. The effects of TNFα on viable cell number of normal and transformed MEC. MEC, isolated from either normal control mammary glands or from NMU-induced mammary tumors, were cultured in complete, serum-free medium (CM, containing 10 ng/ml EGF) in either the presence or absence of 40 ng/ml TNFα. TNFα significantly stimulated the growth of the normal, control MEC, but had only a minimal growth stimulatory effect on the transformed MEC

in this medium condition. Each data point represents the mean ± SEM of triplicate culture wells. This graph is representative of 2 independent experiments.

Figure 29. The effects of TNFα on the morphological appearance of control and NMU-initiated MEC organoids in primary culture. Panels a and b show normal MEC, while panels c and d show NMU-initiated MEC. a, multilobular alveolar and squamous epithelial organoids, 0 EGF medium; b, ductal-alveolar colony network, +40 ng/ml TNFα; c, multilobular alveolar and squamous epithelial organoids, 0 EGF medium; b, ductal-alveolar colony network, +40 ng/ml TNFα. Magnification in all photographs is the same. The magnification bars measure 100 μm.

Figure 30. Analysis of the effects of TNFα on morphological differentiation of control and NMU-initiated MEC organoids in primary culture. The morphologic type of each colony was classified and quantitated on days 13-15 of culture. Four main colony types were quantitated: end bud-like, alveolar, squamous, and atypical hybrid, and the proportion of each is expressed as a percentage of total epithelial colonies. The percentage of squamous and atypical colonies was significantly decreased in all TNFα-treated groups, and the percentage of alveolar colonies was increased when compared to the 0 EGF control. Bars represent the mean ± SEM of triplicate culture wells. This graph is representative of 3 independent experiments.

Figure 31. The effects of TNFα on the morphological appearance of control and transformed MEC organoids in primary culture. Panels a through e show normal control MEC, while panels f through k show transformed MEC. a, squamous epithelial organoids, 0 EGF medium; b, multilobular alveolar organoid, 0 EGF medium; c, ductal-alveolar colony network, +40 ng/ml TNFα; d, multilobular alveolar organoid, complete medium; e, ductal-alveolar colony network, +40 ng/ml TNFα; f, end bud-like, atypical organoids, 0 EGF medium; g and h, multilobular alveolar organoids, +40 ng/ml TNFα; I and j, end bud-like atypical organoids, complete medium; k, multilobular alveolar organoids, +40 ng/ml TNFα. Magnification in all photographs is the same. The magnification bars measure 100 μm.

Figure 32. Analysis of the effects of TNFα on morphological differentiation of control and transformed MEC organoids cultured in EGF-free medium. The morphologic type of each colony was classified and quantitated on days 13-15 of culture. The proportion of each colony type is expressed as a percentage of total epithelial colonies. The percentage of squamous and atypical colonies was significantly decreased in both control and transformed TNFα-treated cultures. The percentage of alveolar colonies was increased when compared to the 0 EGF control; however, a significant percentage of end bud-like colonies was also present in the transformed MEC. Bars represent the mean ± SEM of triplicate culture wells. This graph is representative of 3 independent experiments.

Figure 33. Analysis of the effects of TNFα on morphological differentiation of control and transformed MEC organoids complete medium (CM, containing 10 ng/ml EGF). The morphologic type of each colony was classified and quantitated on days 13-15 of culture, and the proportion of each colony type is expressed as a percentage of total epithelial colonies. While normal, control MEC cultured in this medium are primarily multi-lobular alveolar colonies, the tumor-derived colonies are primarily end bud-like, atypical organoids. The percentage of squamous and atypical colonies was significantly decreased in all TNFα-treated groups. Bars represent the mean ± SEM of triplicate culture wells. This graph is representative of 3 independent experiments.

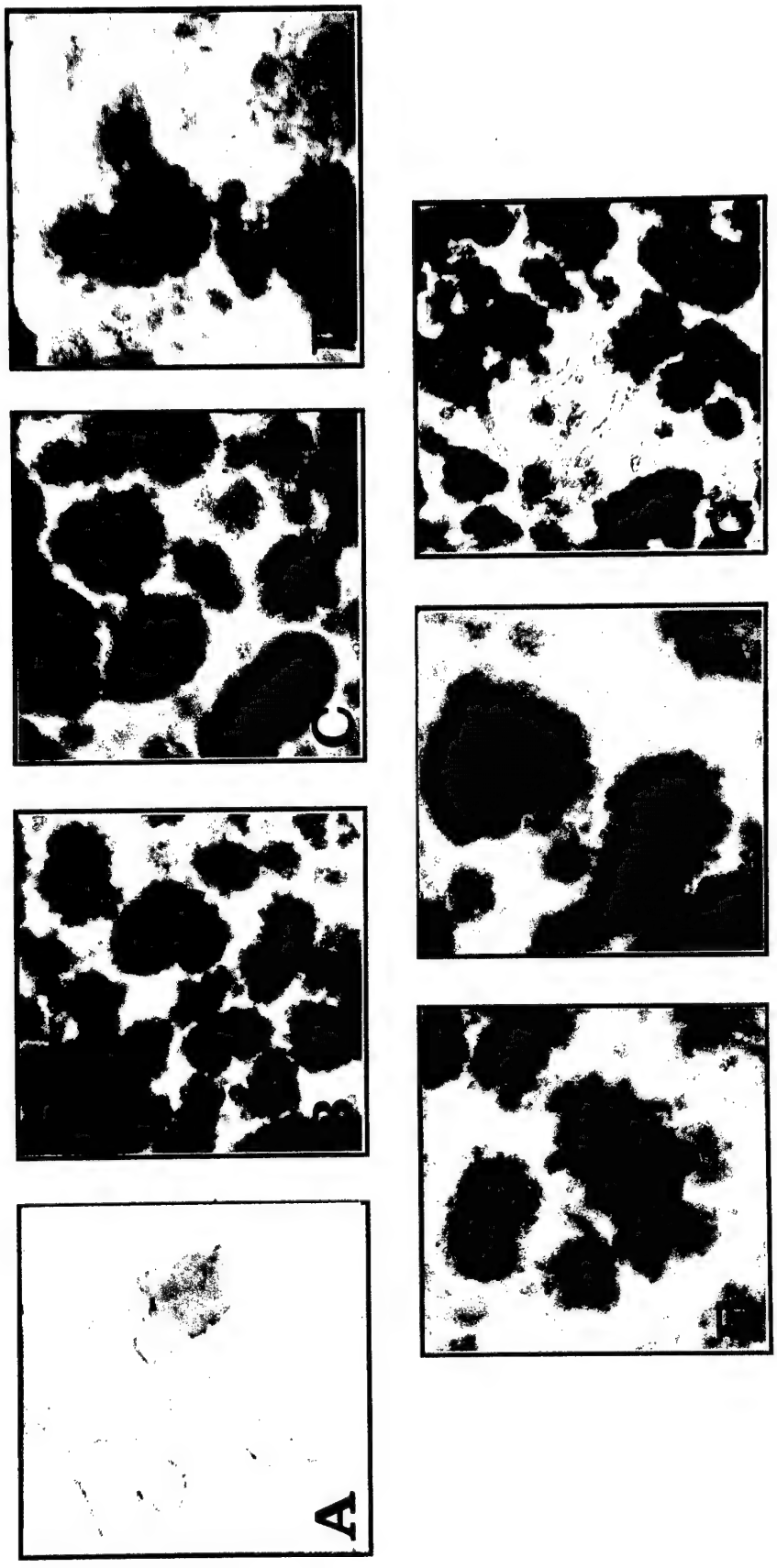


Figure 1

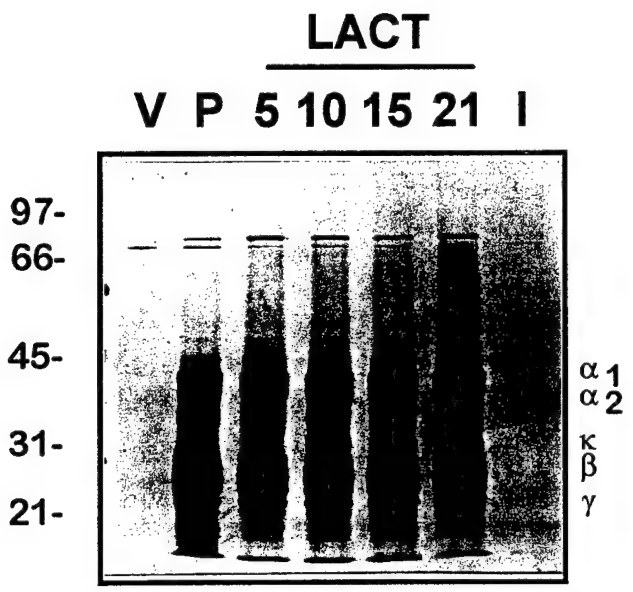


Figure 2

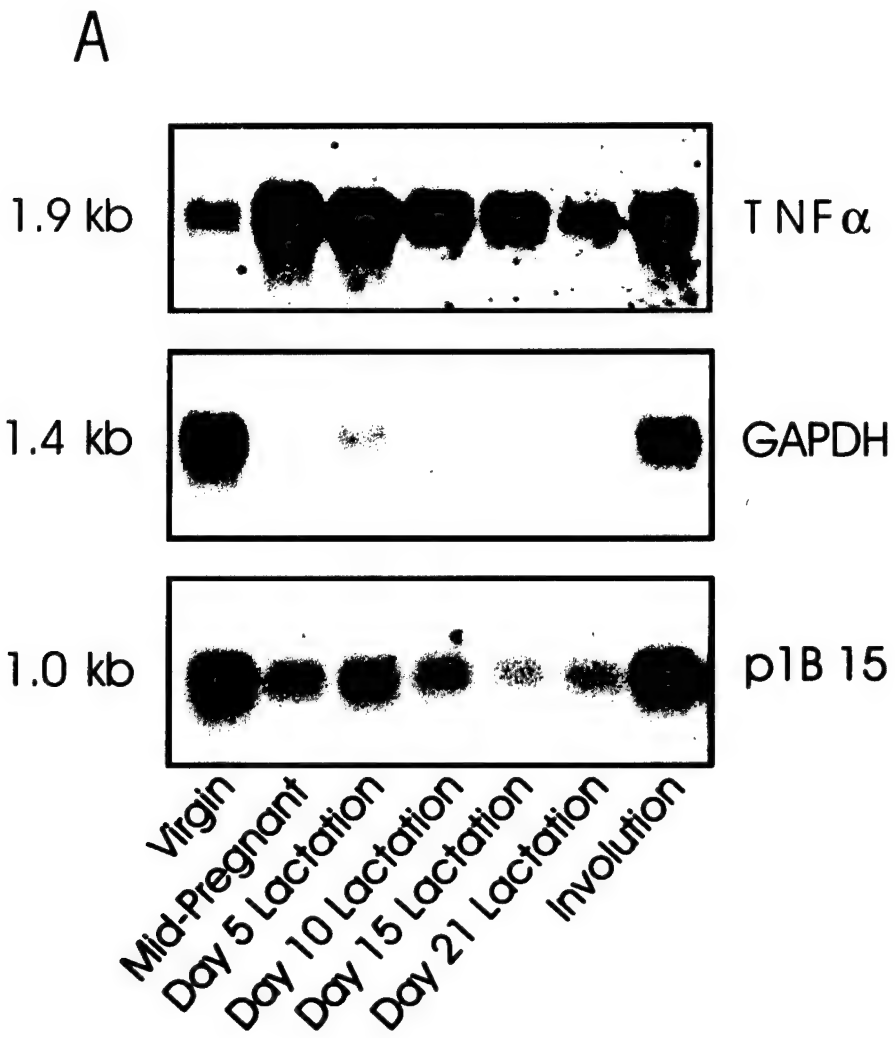
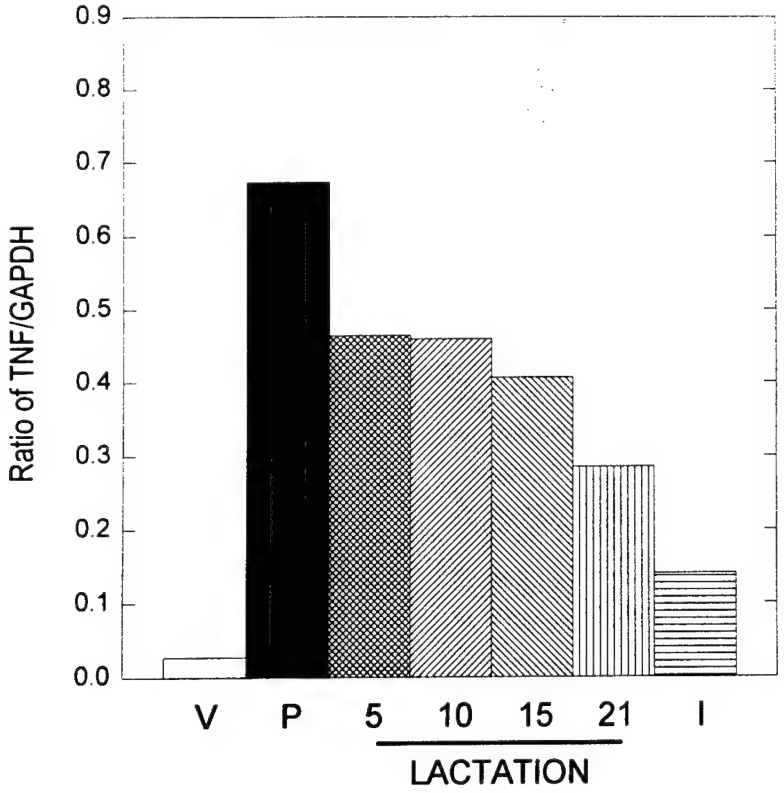
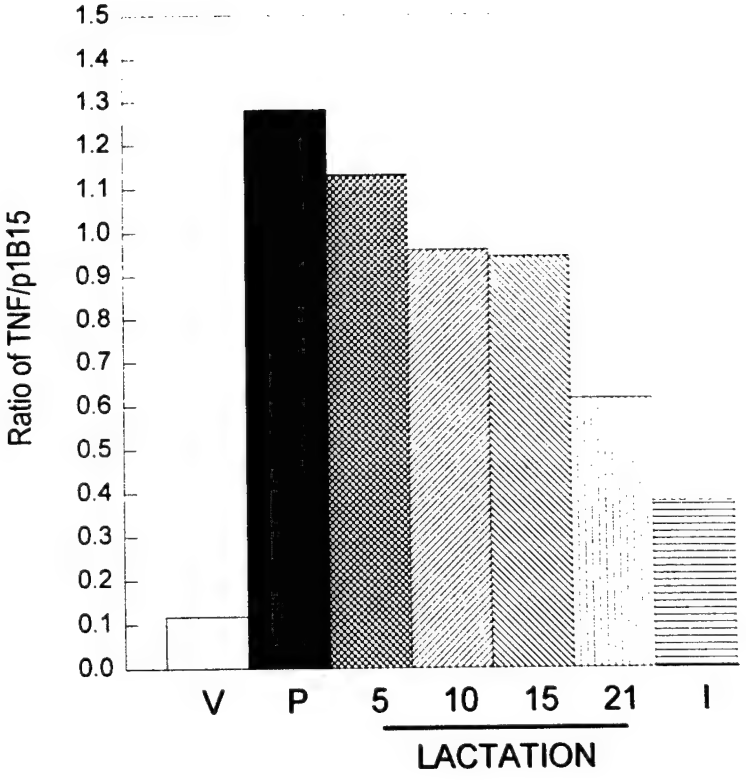

A
1.9 kb
TNFα
1.4 kb
GAPDH
1.0 kb
p1B 15
Virgin
Mid-Pregnant
Day 5 Lactation
Day 10 Lactation
Day 15 Lactation
Day 21 Lactation
Involution

Figure 3A

B



C



Figures 3B and 3C

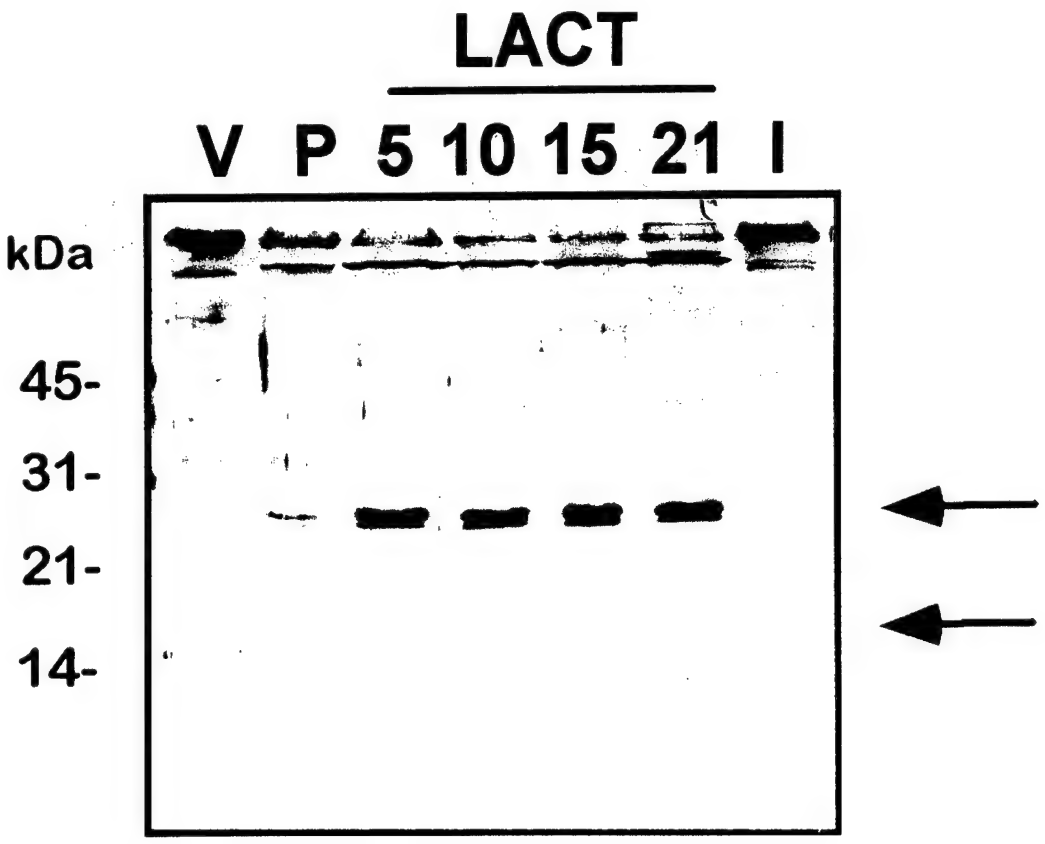
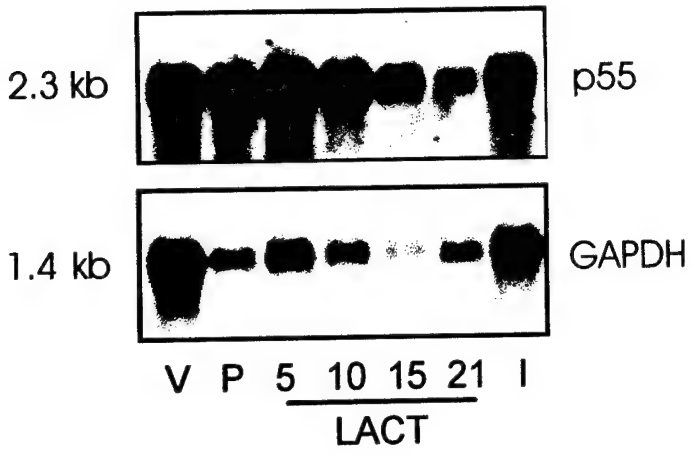


Figure 4

A


2.3 kb
p55
1.4 kb
GAPDH
V P 5 10 15 21 I
LACT

B

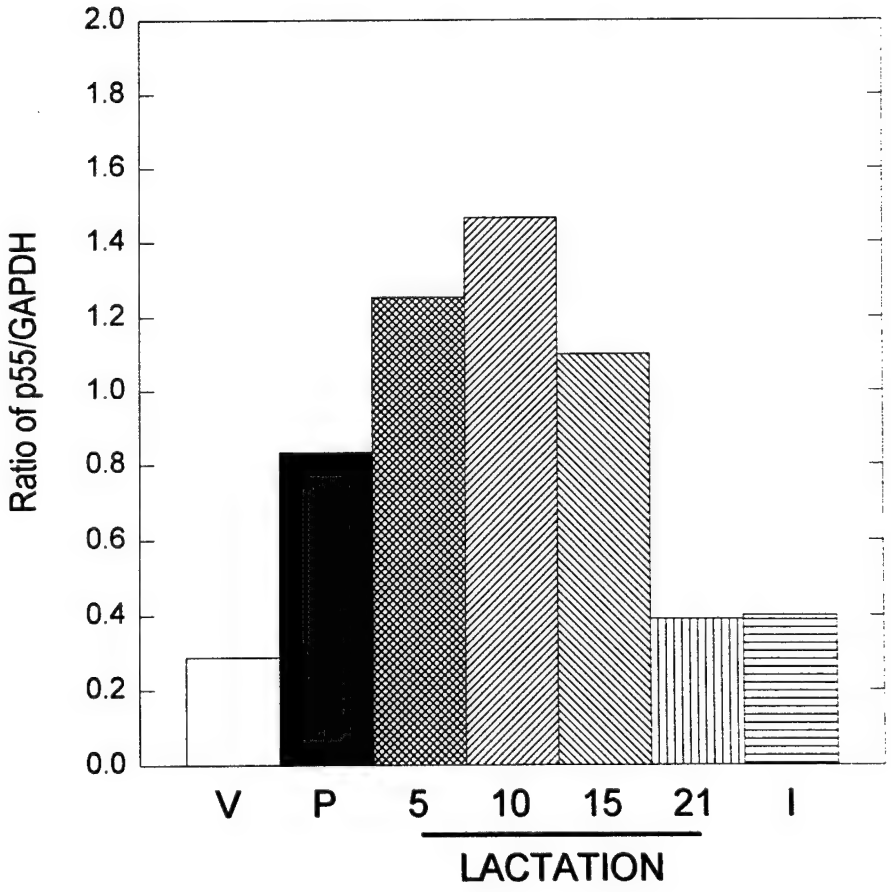
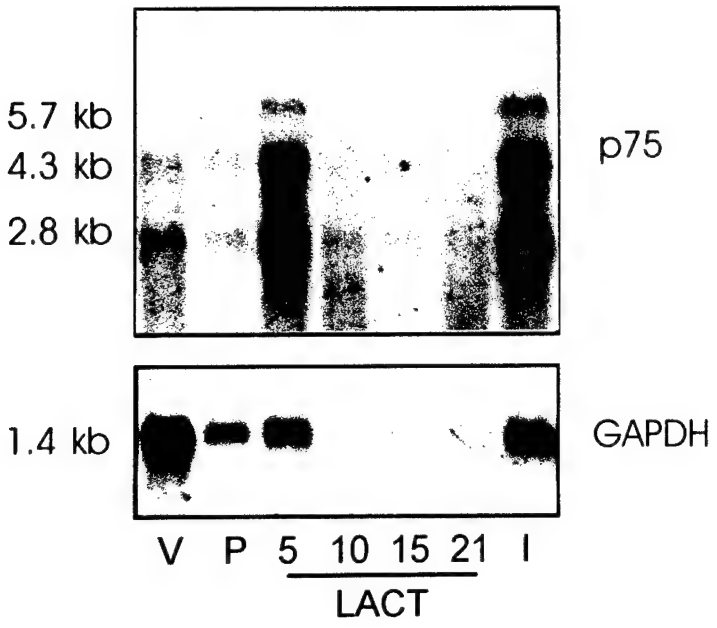

Ratio of p55/GAPDH
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
V P 5 10 15 21 I
LACTATION

Figure 5

A


5.7 kb
4.3 kb
2.8 kb
p75
1.4 kb
GAPDH
V P 5 10 15 21 I
LACT

B

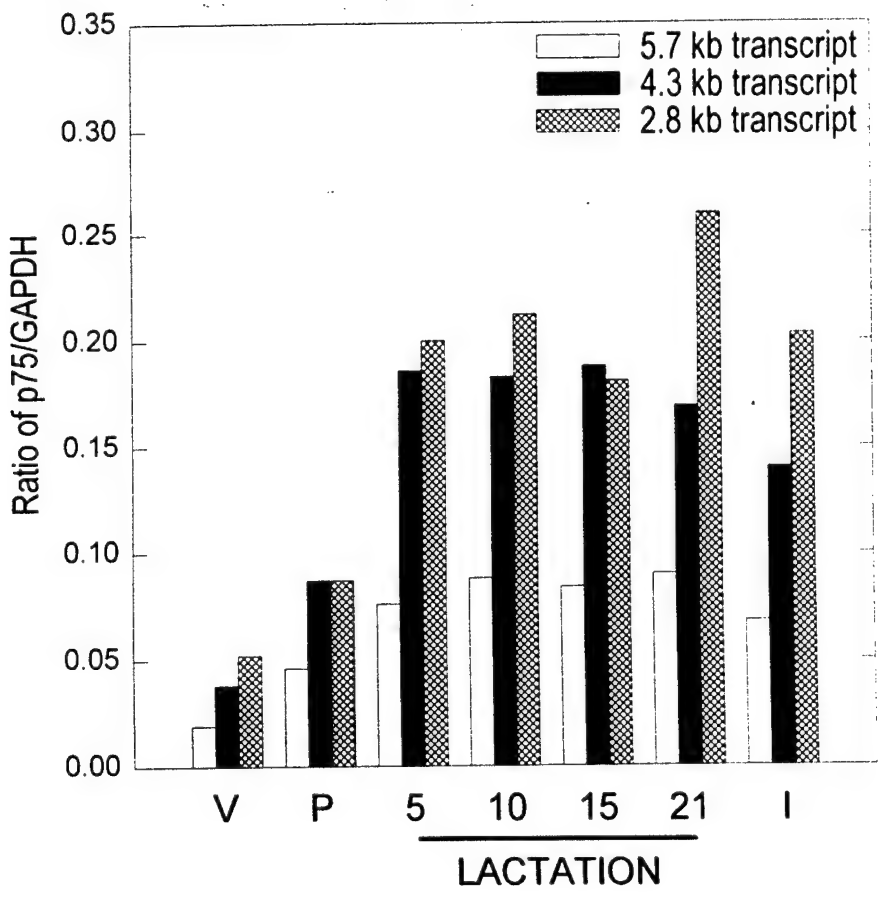
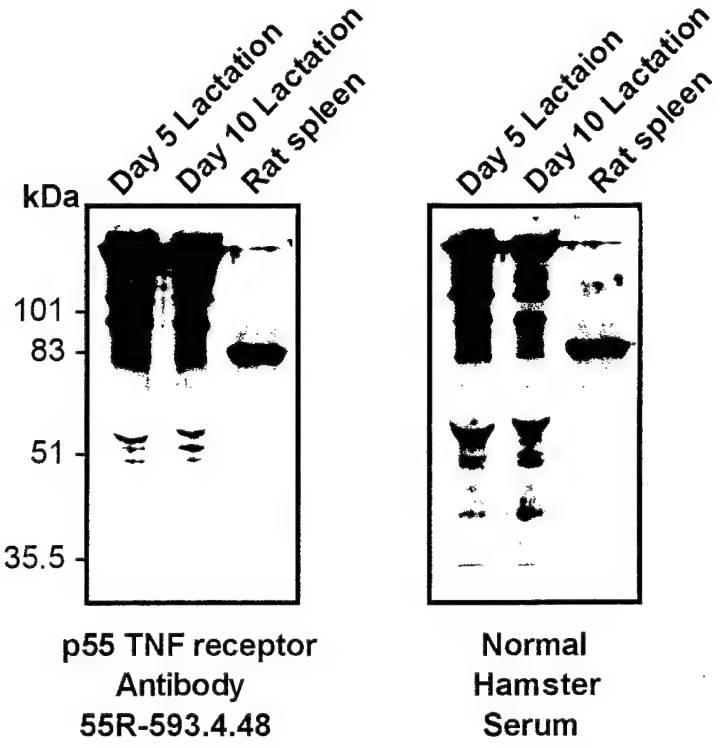

5.7 kb transcript
4.3 kb transcript
2.8 kb transcript
Ratio of p75/GAPDH
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
V P 5 10 15 21 I
LACTATION

Figure 6



p55 TNF receptor Antibody 55R-593.4.48

Normal Hamster Serum

Figure 7

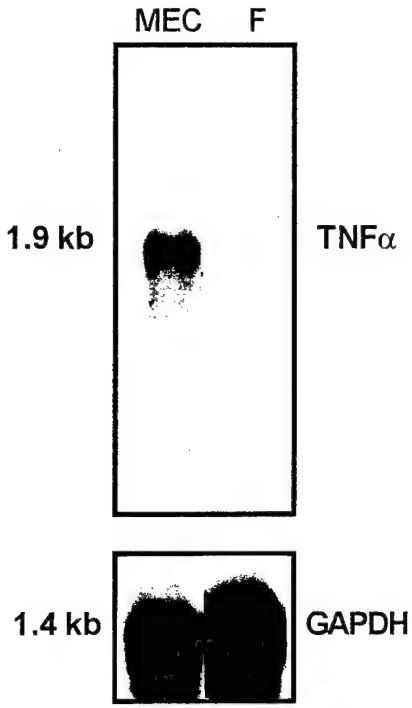


Figure 8

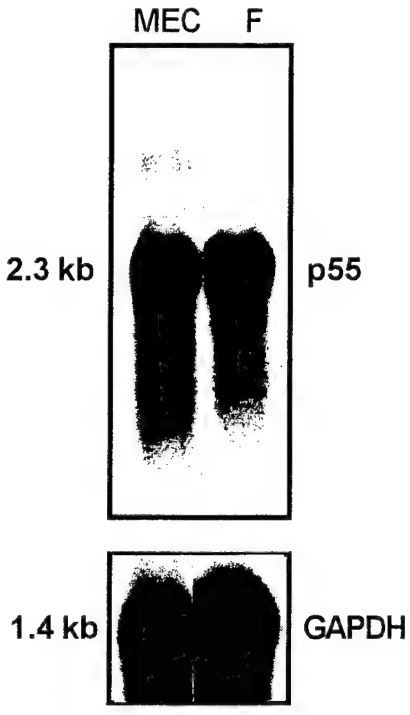


Figure 9

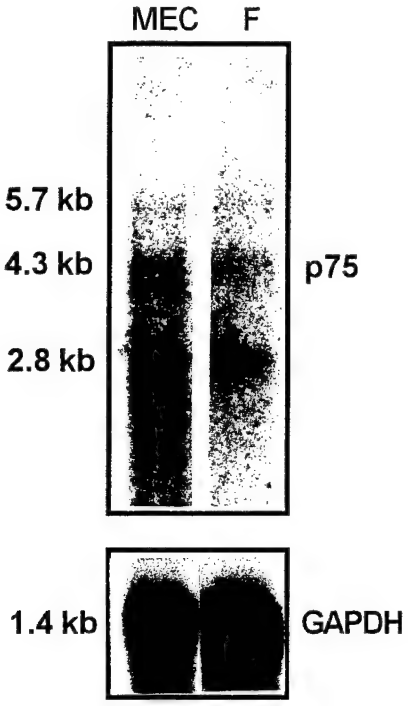


Figure 10

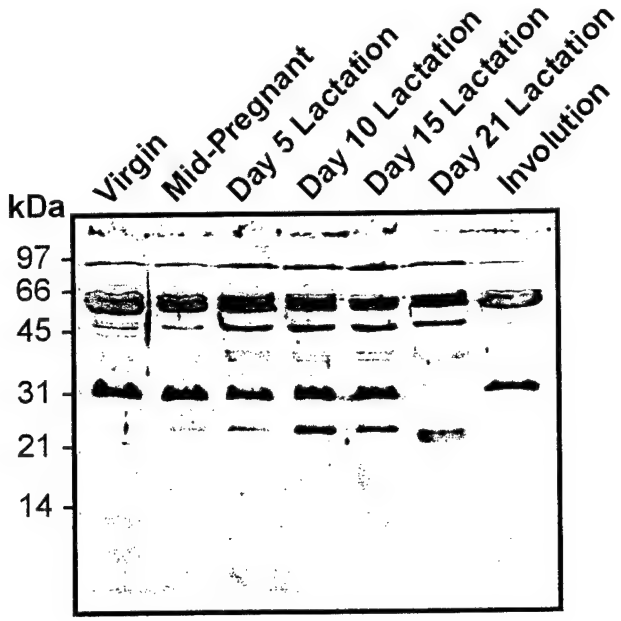

Virgin
Mid-Pregnant
Day 5 Lactation
Day 10 Lactation
Day 15 Lactation
Day 21 Lactation
Involution
kDa
97
66
45
31
21
14

Figure 11

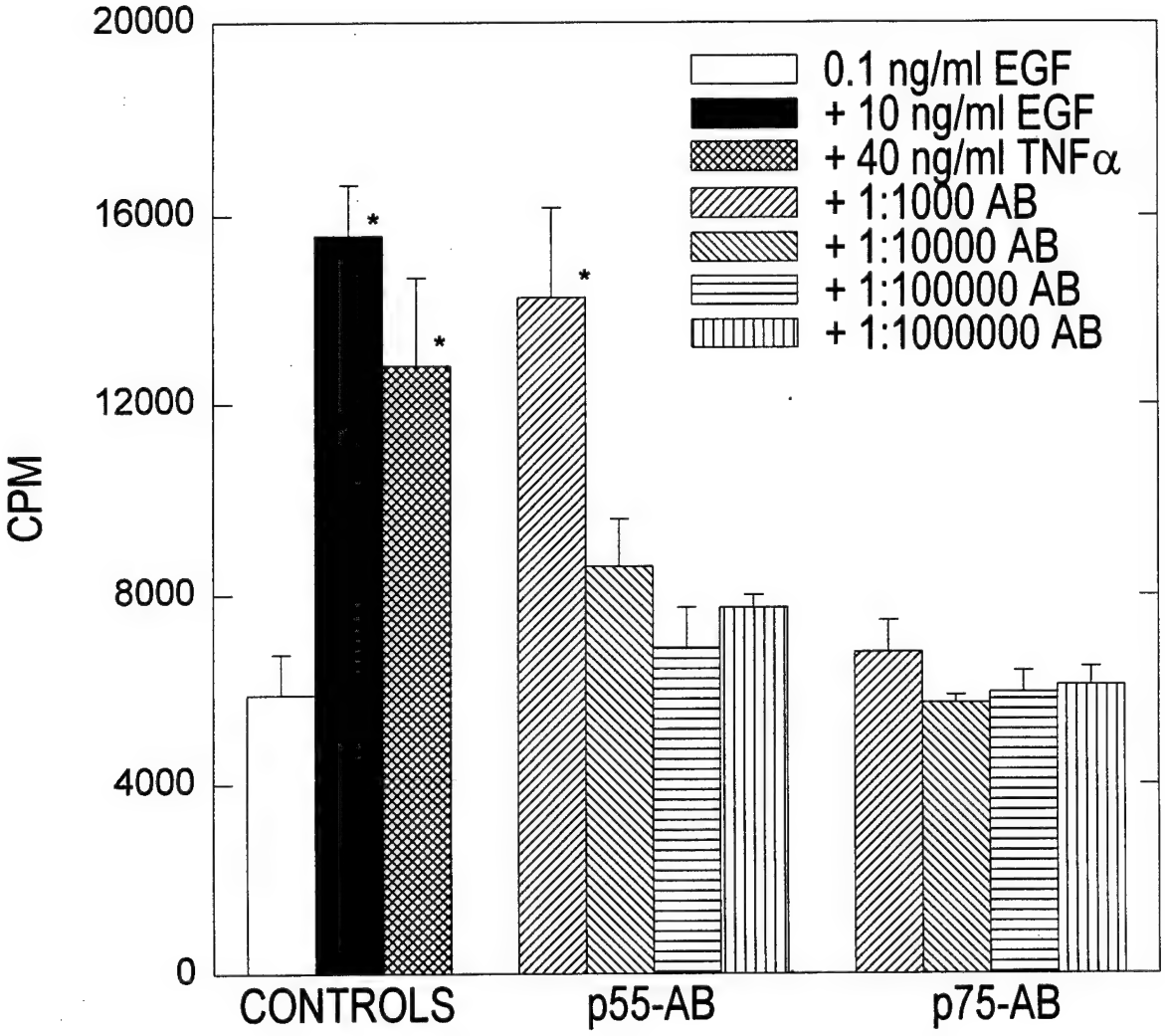

20000
16000
12000
8000
4000
0
CPM
0.1 ng/ml EGF
+ 10 ng/ml EGF
+ 40 ng/ml TNFα
+ 1:1000 AB
+ 1:10000 AB
+ 1:100000 AB
+ 1:1000000 AB
*
*
*
CONTROLS
p55-AB
p75-AB

Figure 12

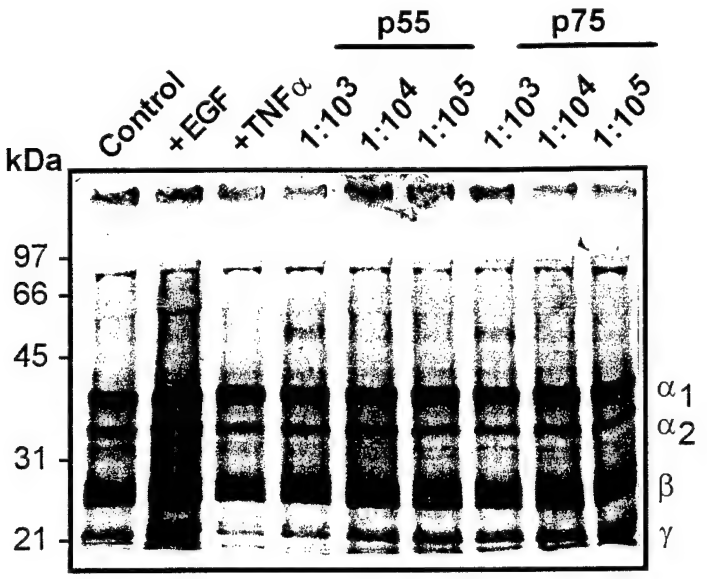

p55
p75
Control
+EGF
+TNFα
1:10³
1:10⁴
1:10⁵
1:10³
1:10⁴
1:10⁵
kDa
97
66
45
31
21
α1
α2
β
γ

Figure 13A

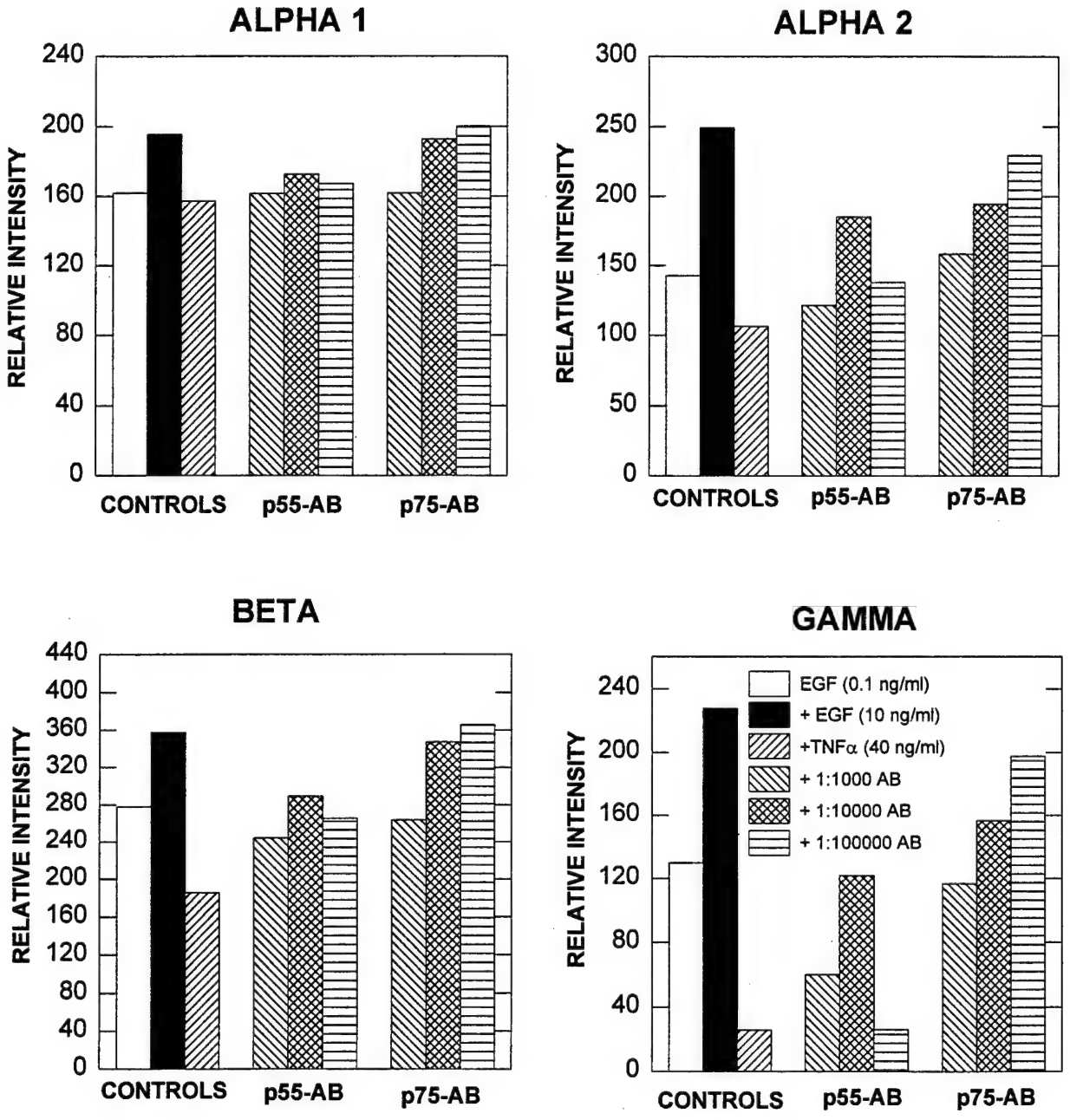

ALPHA 1
RELATIVE INTENSITY
240
200
160
120
80
40
0
CONTROLS
p55-AB
p75-AB
ALPHA 2
RELATIVE INTENSITY
300
250
200
150
100
50
0
CONTROLS
p55-AB
p75-AB
BETA
RELATIVE INTENSITY
440
400
360
320
280
240
200
160
120
80
40
0
CONTROLS
p55-AB
p75-AB
GAMMA
RELATIVE INTENSITY
240
200
160
120
80
40
0
CONTROLS
p55-AB
p75-AB
EGF (0.1 ng/ml)
+ EGF (10 ng/ml)
+TNFα (40 ng/ml)
+ 1:1000 AB
+ 1:10000 AB
+ 1:100000 AB

Figure 13B

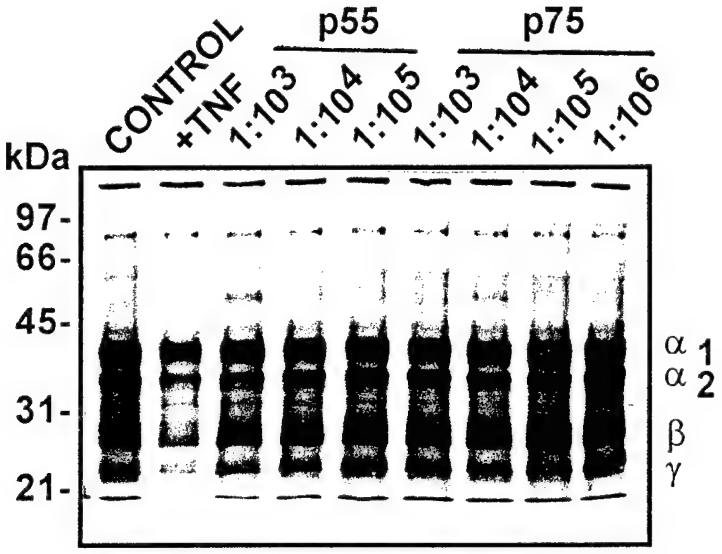

p55
p75
CONTROL
+TNF
1:10³
1:10⁴
1:10⁵
1:10³
1:10⁴
1:10⁵
1:10⁶
kDa
97-
66-
45-
31-
21-
α 1
α 2
β
γ

Figure 14A

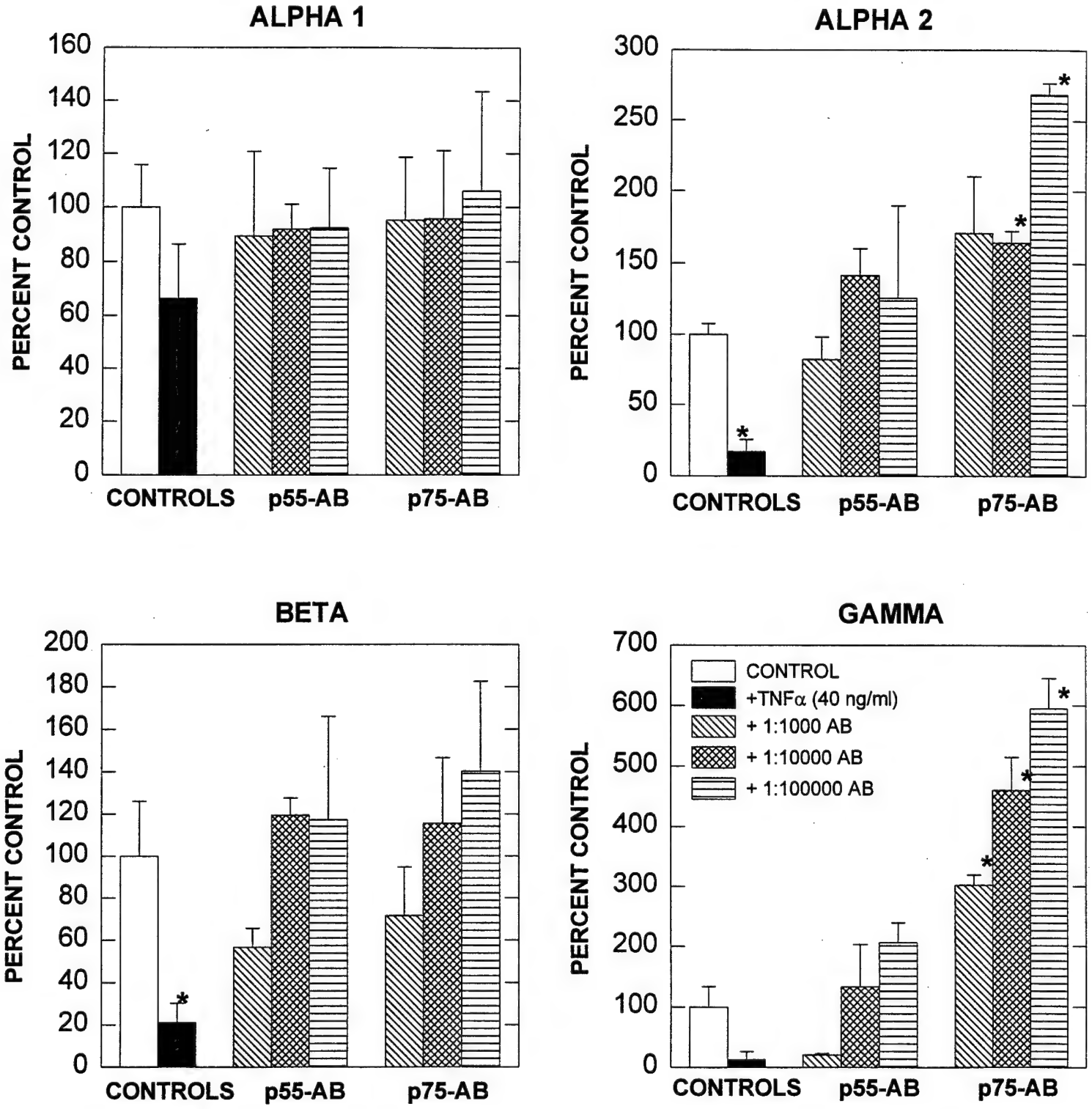

ALPHA 1
PERCENT CONTROL
160
140
120
100
80
60
40
20
0
CONTROLS
p55-AB
p75-AB
ALPHA 2
PERCENT CONTROL
300
250
200
150
100
50
0
CONTROLS
p55-AB
p75-AB
BETA
PERCENT CONTROL
200
180
160
140
120
100
80
60
40
20
0
CONTROLS
p55-AB
p75-AB
GAMMA
PERCENT CONTROL
700
600
500
400
300
200
100
0
CONTROL
+TNFα (40 ng/ml)
+ 1:1000 AB
+ 1:10000 AB
+ 1:100000 AB
CONTROLS
p55-AB
p75-AB

Figure 14B

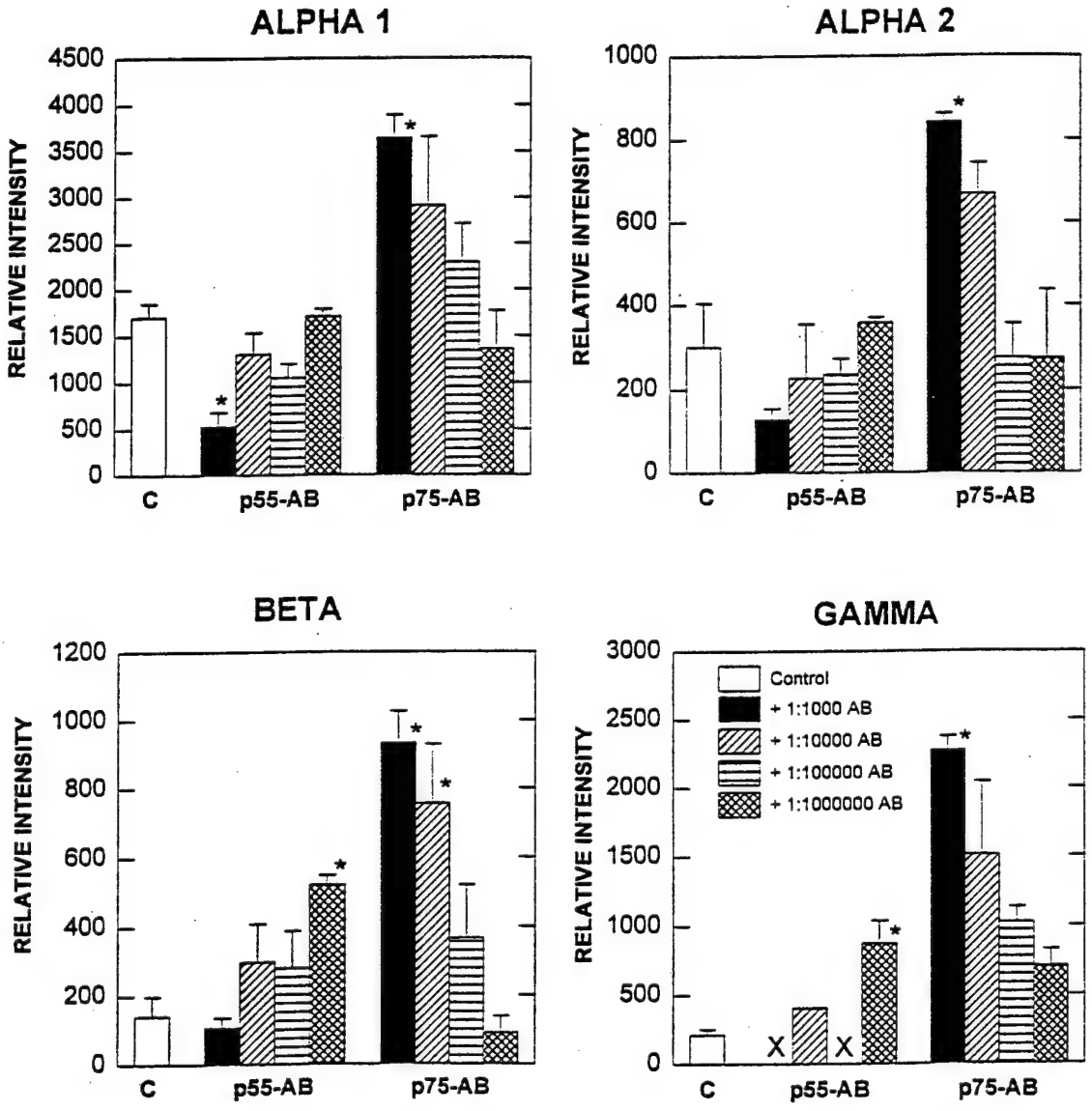

ALPHA 1
RELATIVE INTENSITY
4500
4000
3500
3000
2500
2000
1500
1000
500
0
C
p55-AB
p75-AB
ALPHA 2
RELATIVE INTENSITY
1000
800
600
400
200
0
C
p55-AB
p75-AB
BETA
RELATIVE INTENSITY
1200
1000
800
600
400
200
0
C
p55-AB
p75-AB
GAMMA
RELATIVE INTENSITY
3000
2500
2000
1500
1000
500
0
Control
+ 1:1000 AB
+ 1:10000 AB
+ 1:100000 AB
+ 1:1000000 AB
C
p55-AB
p75-AB

Figure 15

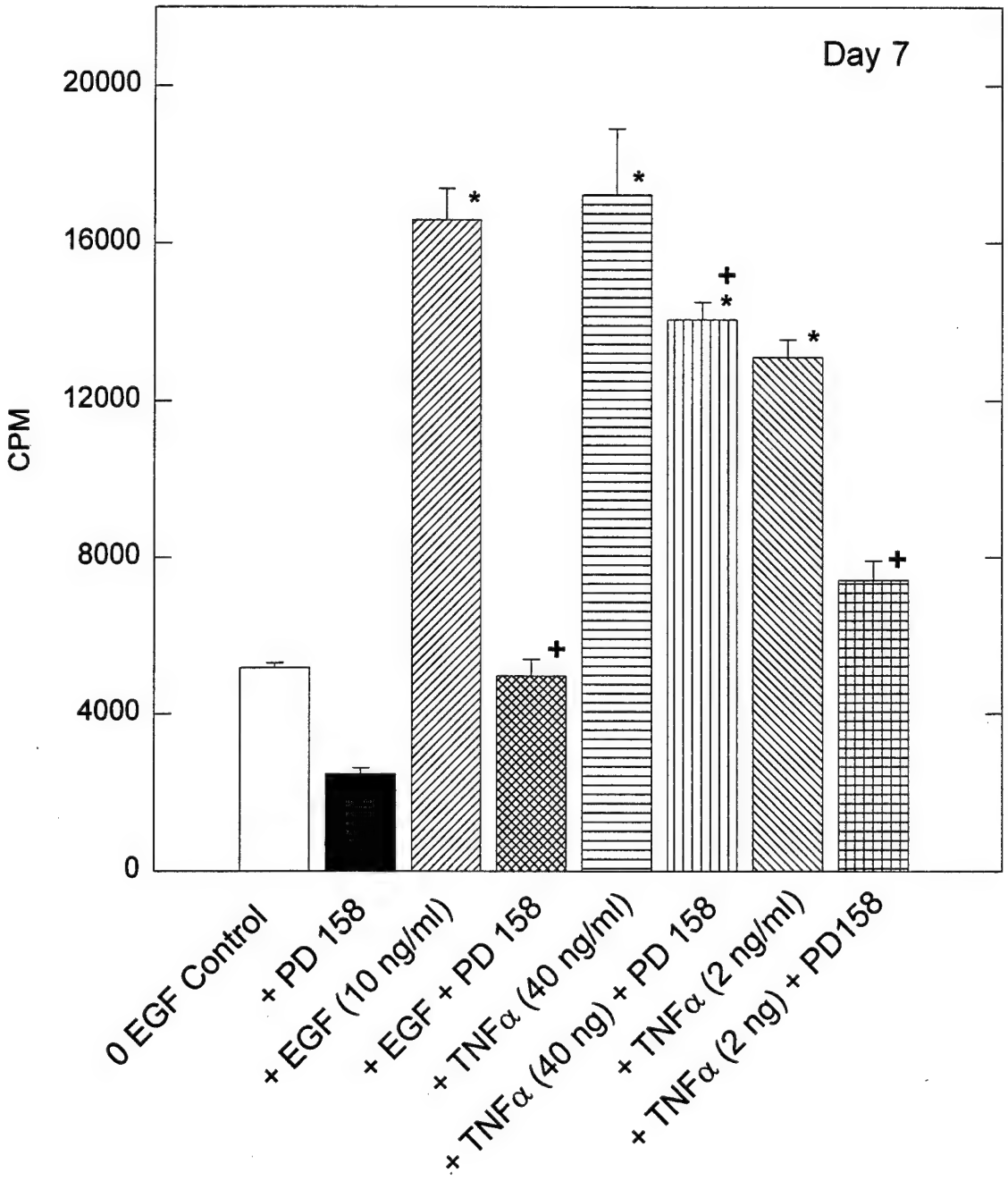

Day 7
CPM
20000
16000
12000
8000
4000
0
0 EGF Control
+ PD 158
+ EGF (10 ng/ml)
+ EGF + PD 158
+ TNFα (40 ng/ml)
+ TNFα (40 ng) + PD 158
+ TNFα (2 ng/ml)
+ TNFα (2 ng) + PD158

Figure 16

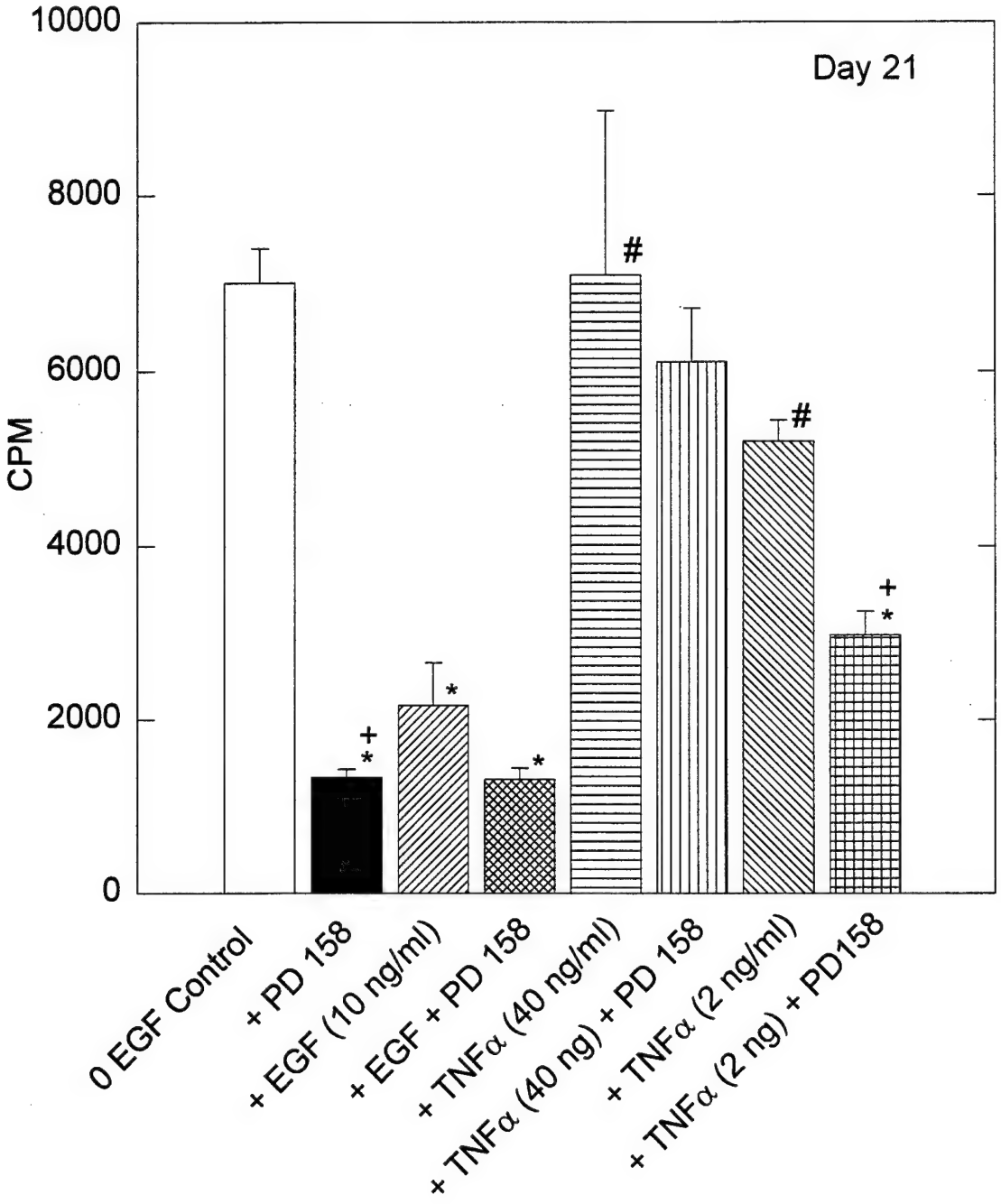

Day 21
CPM
10000
8000
6000
4000
2000
0
#
#
+
*
*
*
+
*
0 EGF Control
+ PD 158
+ EGF (10 ng/ml)
+ EGF + PD 158
+ TNFα (40 ng/ml)
+ TNFα (40 ng) + PD 158
+ TNFα (2 ng/ml)
+ TNFα (2 ng) + PD158

Figure 17

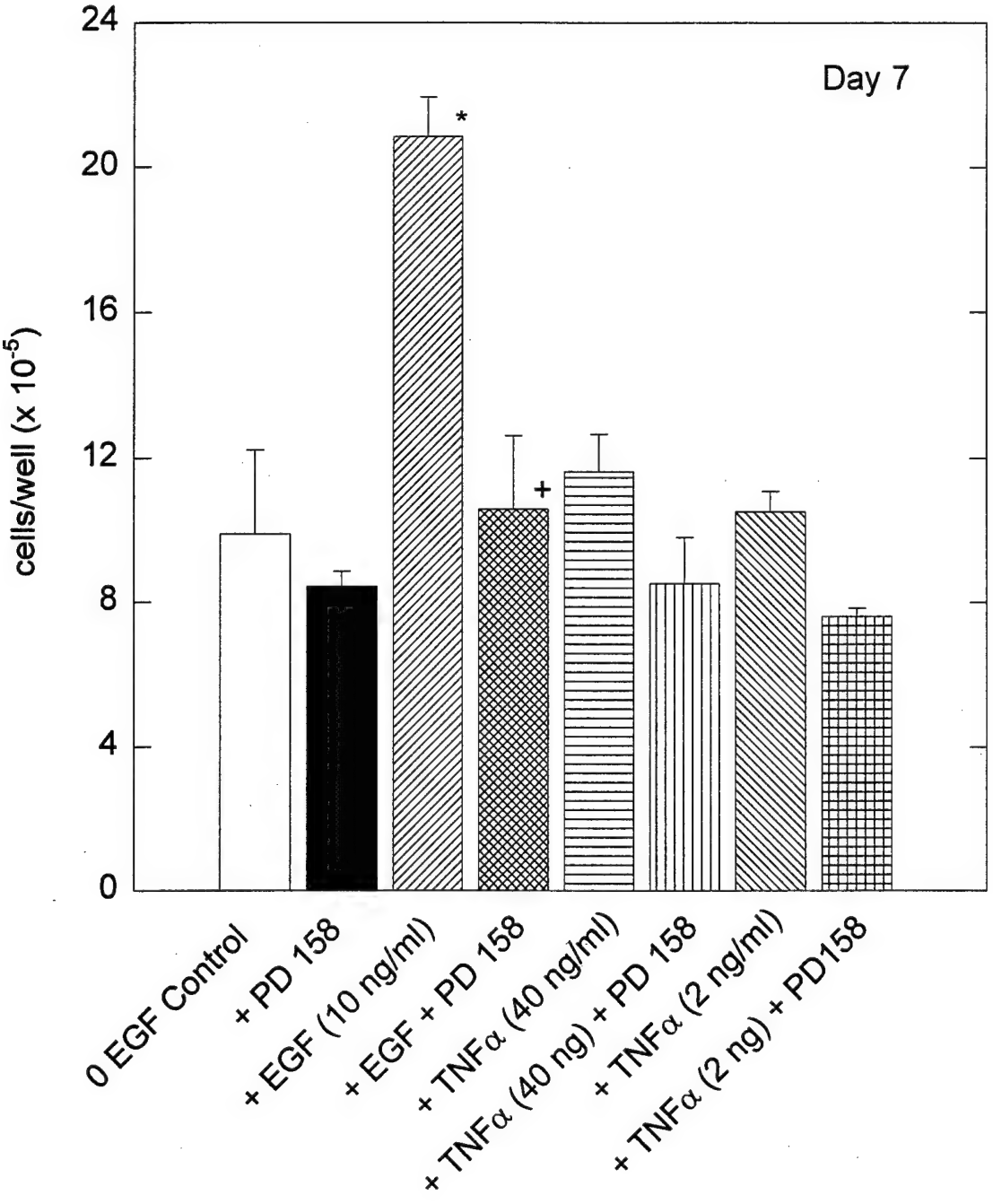

Day 7
cells/well (x 10^{-5})
24
20
16
12
8
4
0
*
+
0 EGF Control
+ PD 158
+ EGF (10 ng/ml)
+ EGF + PD 158
+ TNFα (40 ng/ml)
+ TNFα (40 ng) + PD 158
+ TNFα (2 ng/ml)
+ TNFα (2 ng) + PD158

Figure 18

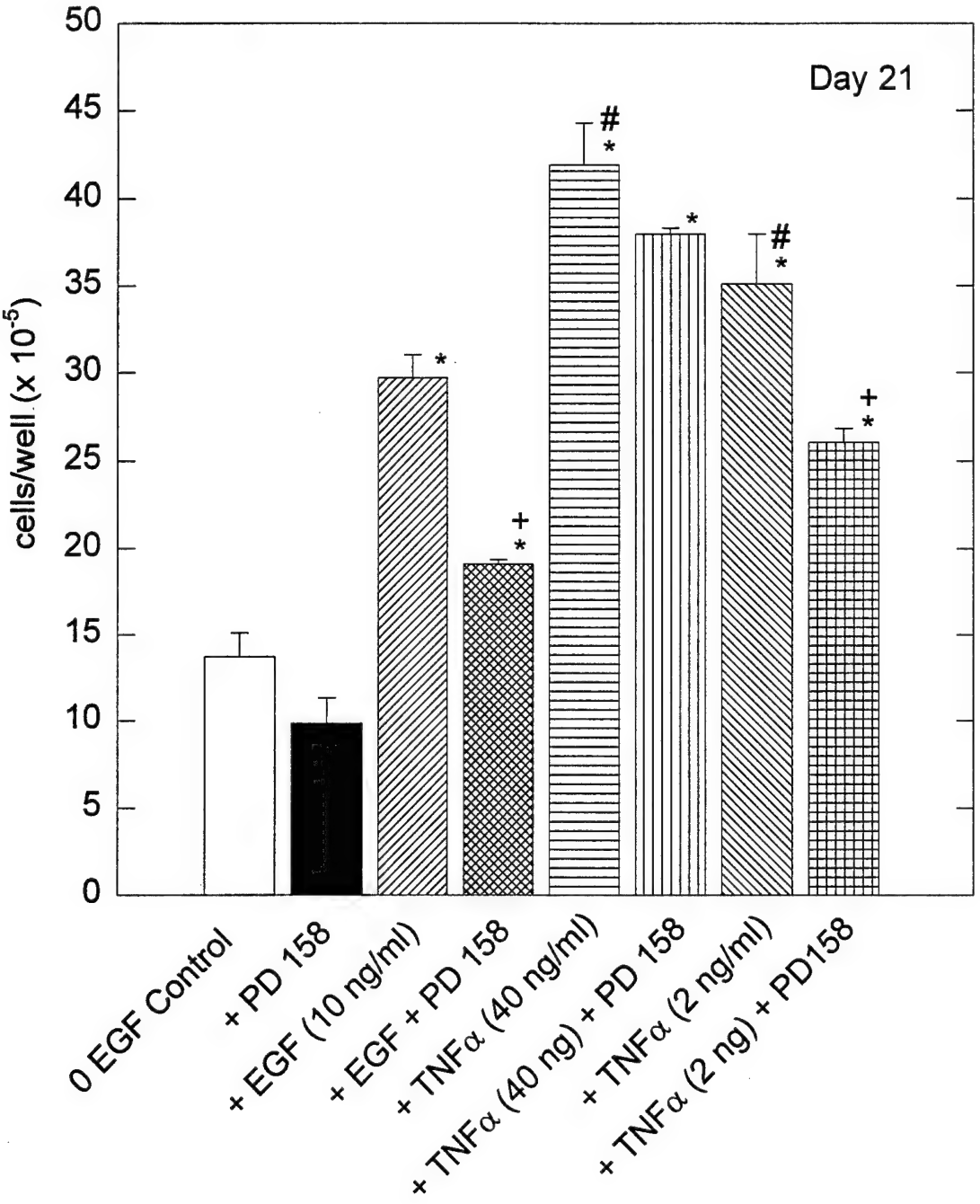

Day 21
cells/well (x 10-5)
50
45
40
35
30
25
20
15
10
5
0
0 EGF Control
+ PD 158
+ EGF (10 ng/ml)
+ EGF + PD 158
+ TNFα (40 ng/ml)
+ TNFα (40 ng) + PD 158
+ TNFα (2 ng/ml)
+ TNFα (2 ng) + PD158

Figure 19

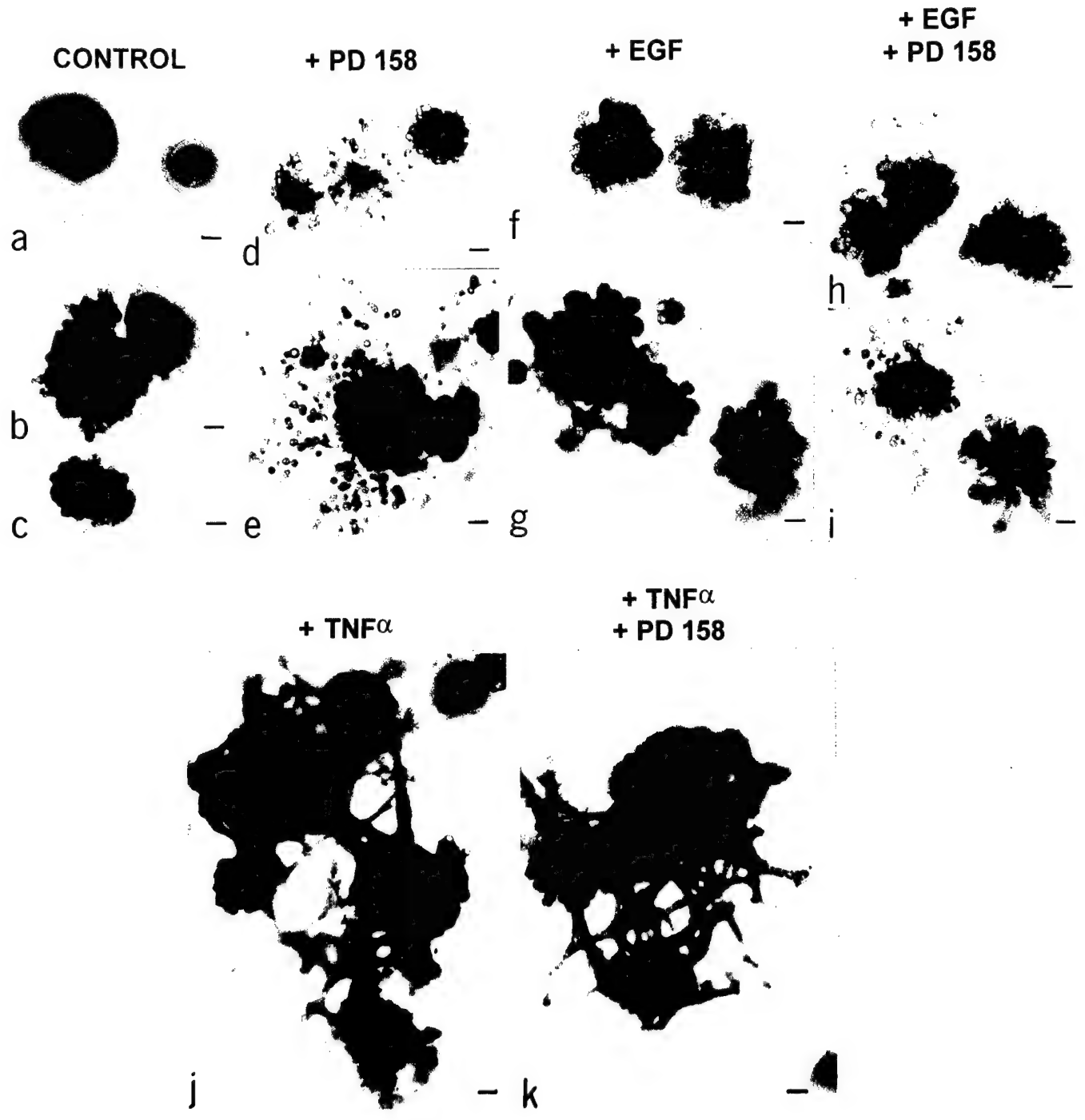


Figure 20

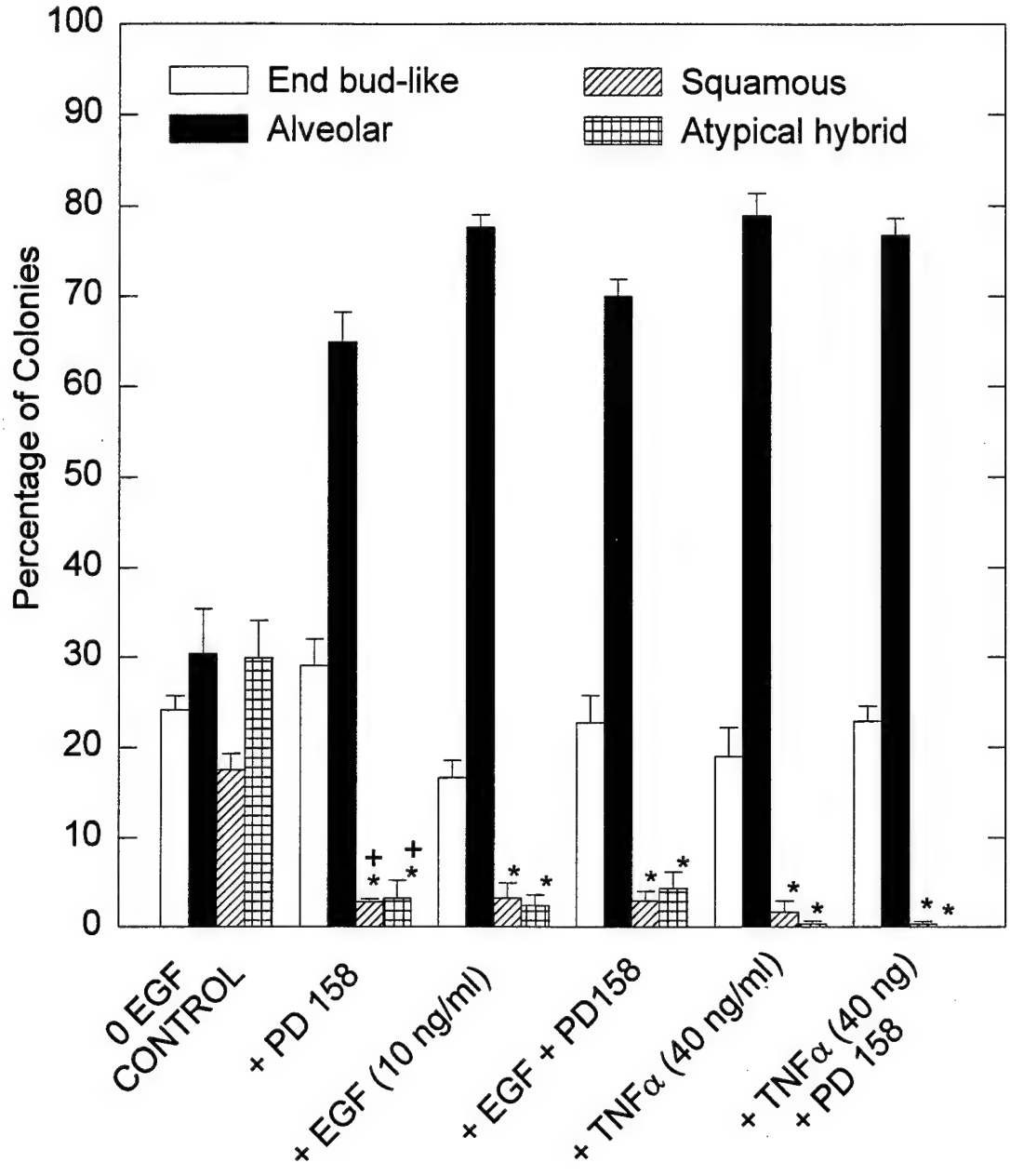

End bud-like
Alveolar
Squamous
Atypical hybrid
Percentage of Colonies
100
90
80
70
60
50
40
30
20
10
0
0 EGF CONTROL
+ PD 158
+ EGF (10 ng/ml)
+ EGF + PD158
+ TNFα (40 ng/ml)
+ TNFα (40 ng) + PD 158

Figure 21

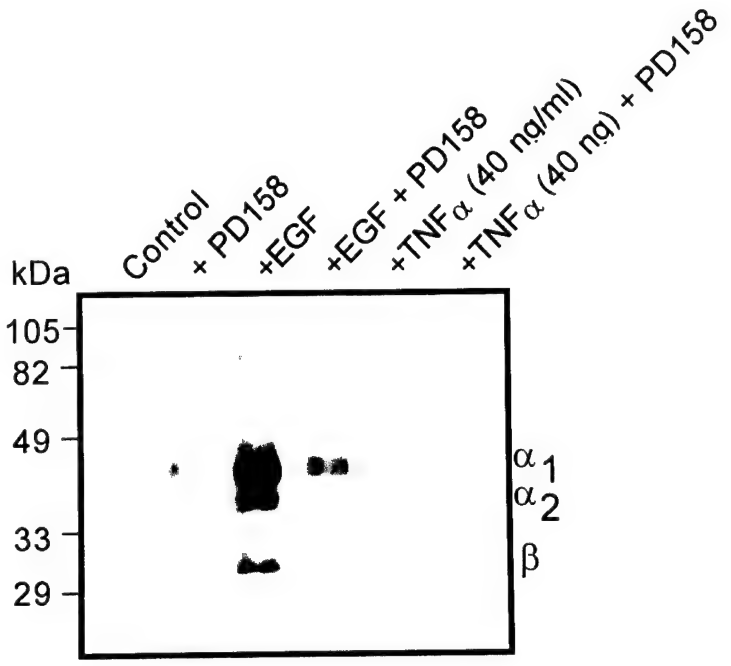

Control
+ PD158
+EGF
+EGF + PD158
+TNFα (40 ng/ml)
+TNFα (40 ng) + PD158
kDa
105
82
49
33
29
α1
α2
β

Figure 22

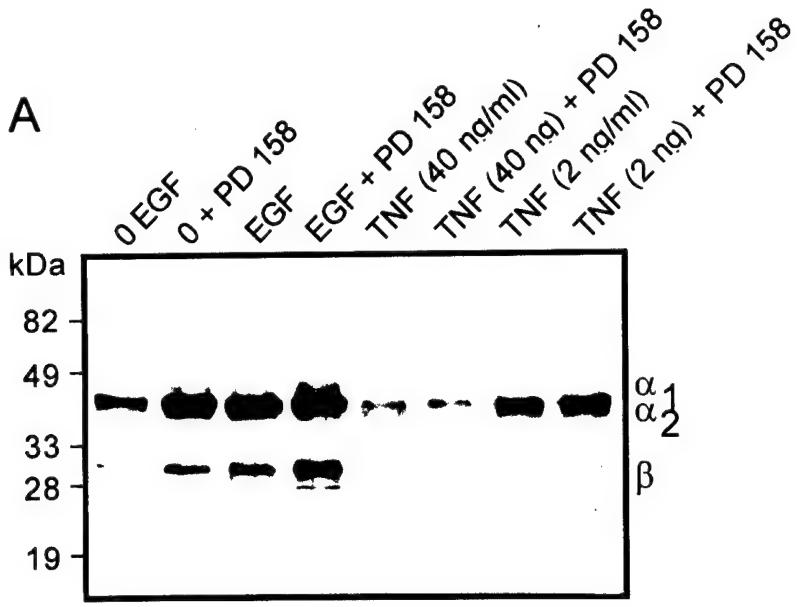

A
0 EGF
0 + PD 158
EGF
EGF + PD 158
TNF (40 ng/ml)
TNF (40 ng) + PD 158
TNF (2 ng/ml)
TNF (2 ng) + PD 158
kDa
82
49
33
28
19
α1
α2
β

Figure 23A

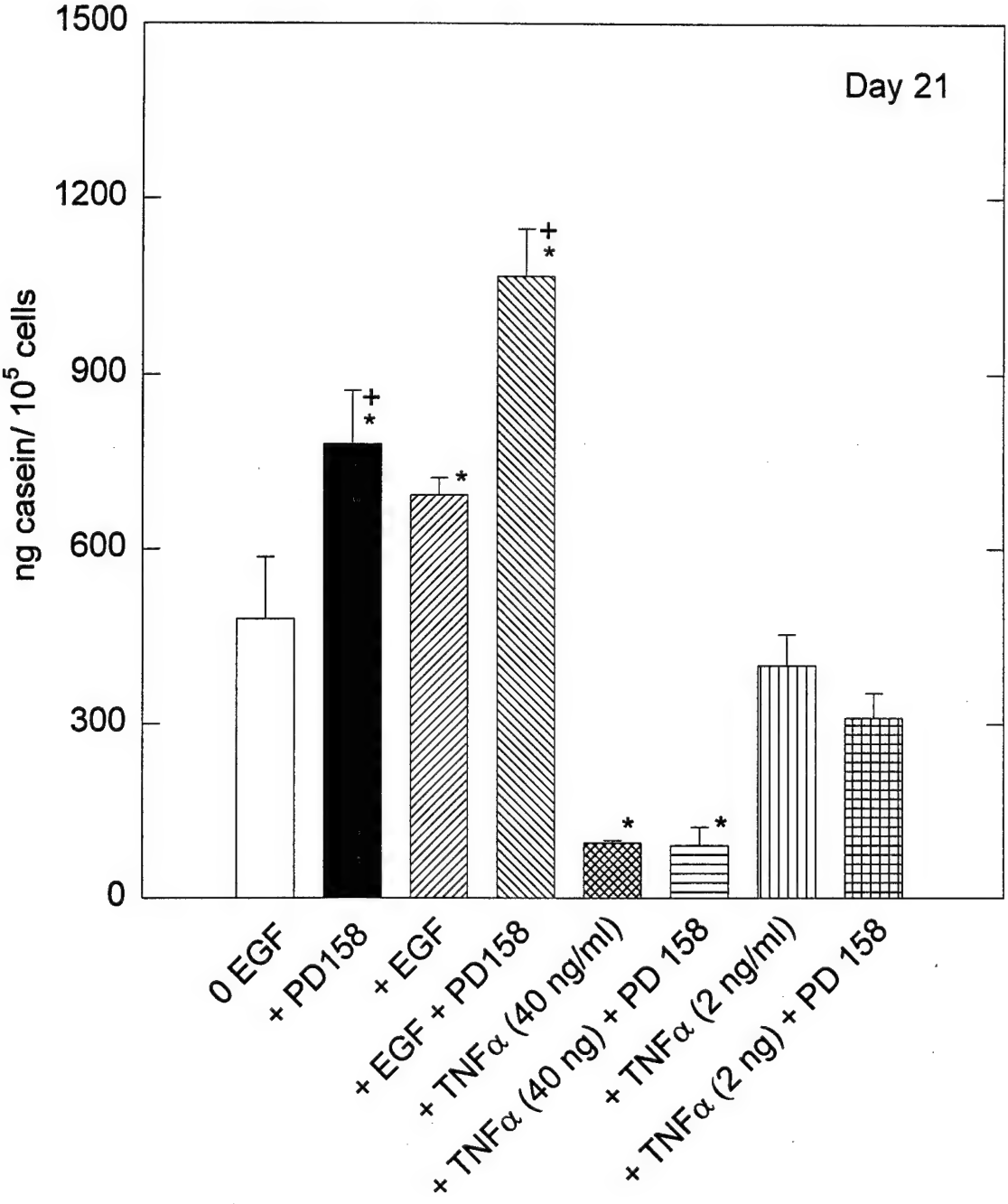
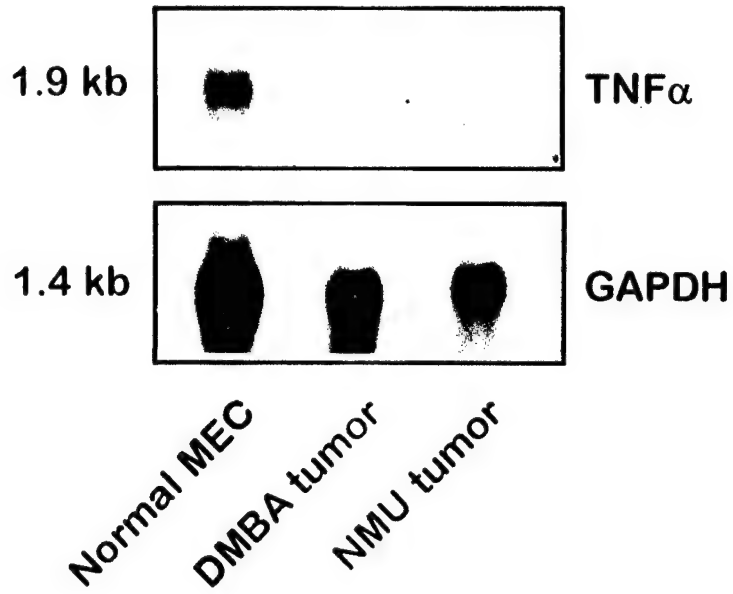

Day 21
ng casein/ 10^5 cells
1500
1200
900
600
300
0
0 EGF
+ PD158
+ EGF
+ EGF + PD158
+ TNFα (40 ng/ml)
+ TNFα (40 ng) + PD 158
+ TNFα (2 ng/ml)
+ TNFα (2 ng) + PD 158

Figure 23B


1.9 kb
TNFα
1.4 kb
GAPDH
Normal MEC
DMBA tumor
NMU tumor

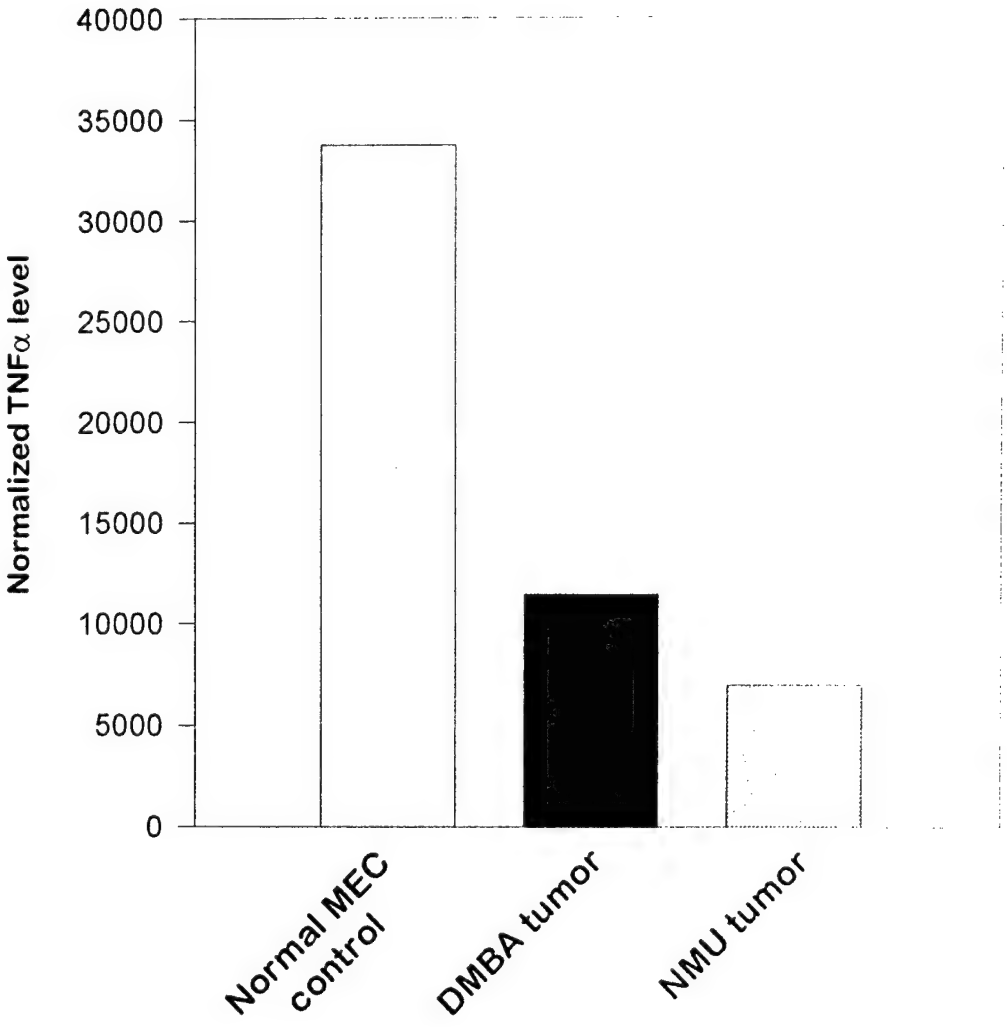

Normalized TNFα level
40000
35000
30000
25000
20000
15000
10000
5000
0
Normal MEC control
DMBA tumor
NMU tumor

Figure 24

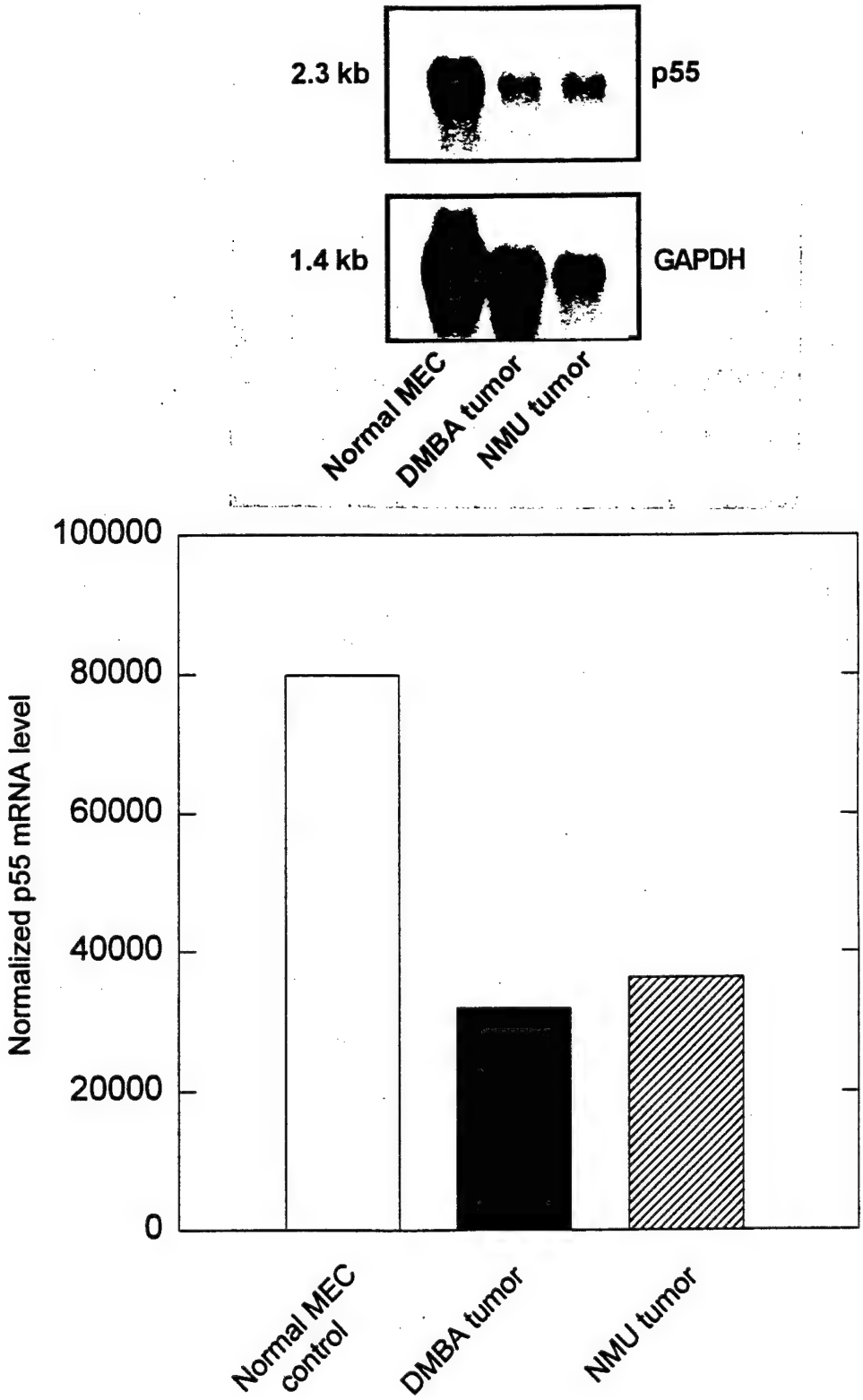

2.3 kb
p55
1.4 kb
GAPDH
Normal MEC
DMBA tumor
NMU tumor
100000
80000
60000
40000
20000
0
Normalized p55 mRNA level
Normal MEC control
DMBA tumor
NMU tumor

Figure 25

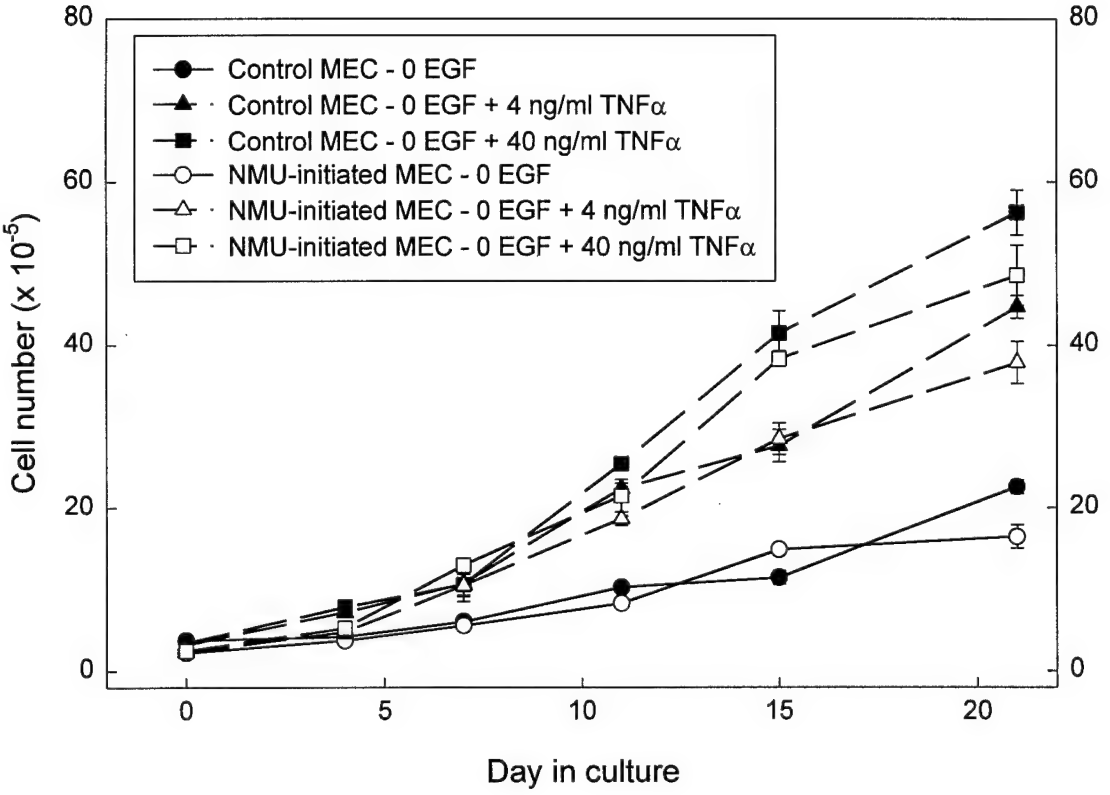

Control MEC - 0 EGF
Control MEC - 0 EGF + 4 ng/ml TNFα
Control MEC - 0 EGF + 40 ng/ml TNFα
NMU-initiated MEC - 0 EGF
NMU-initiated MEC - 0 EGF + 4 ng/ml TNFα
NMU-initiated MEC - 0 EGF + 40 ng/ml TNFα
Cell number (x 10-5)
Day in culture
0
20
40
60
80
0
5
10
15
20

Figure 26

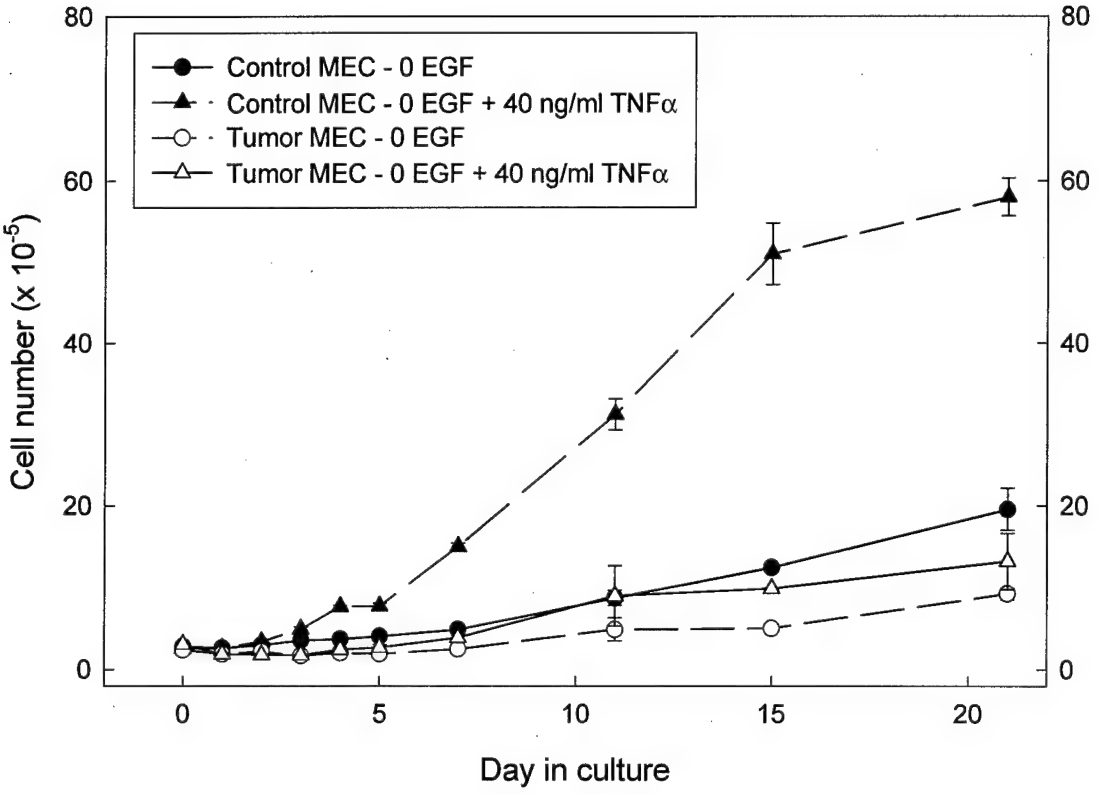

Control MEC - 0 EGF
Control MEC - 0 EGF + 40 ng/ml TNFα
Tumor MEC - 0 EGF
Tumor MEC - 0 EGF + 40 ng/ml TNFα
Cell number (x 10-5)
Day in culture
0
20
40
60
80
0
5
10
15
20

Figure 27

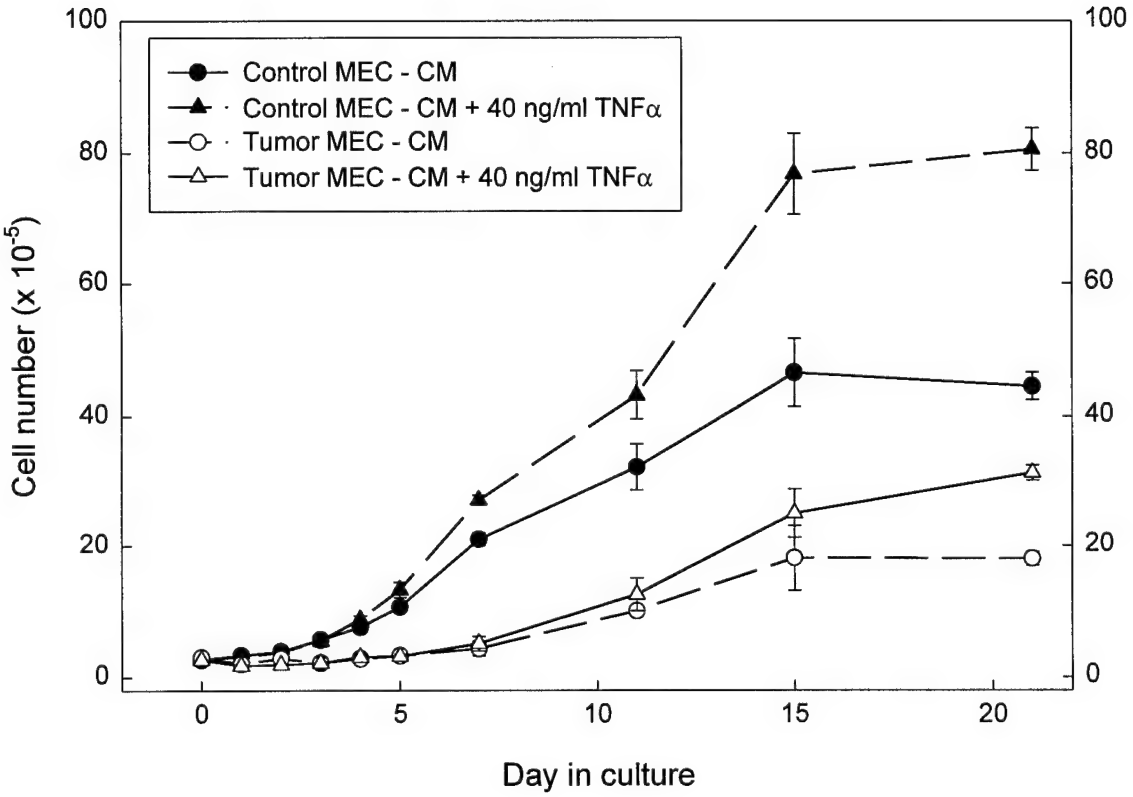

Control MEC - CM
Control MEC - CM + 40 ng/ml TNFα
Tumor MEC - CM
Tumor MEC - CM + 40 ng/ml TNFα
Cell number (x 10-5)
Day in culture
0
20
40
60
80
100
0
5
10
15
20

Figure 28

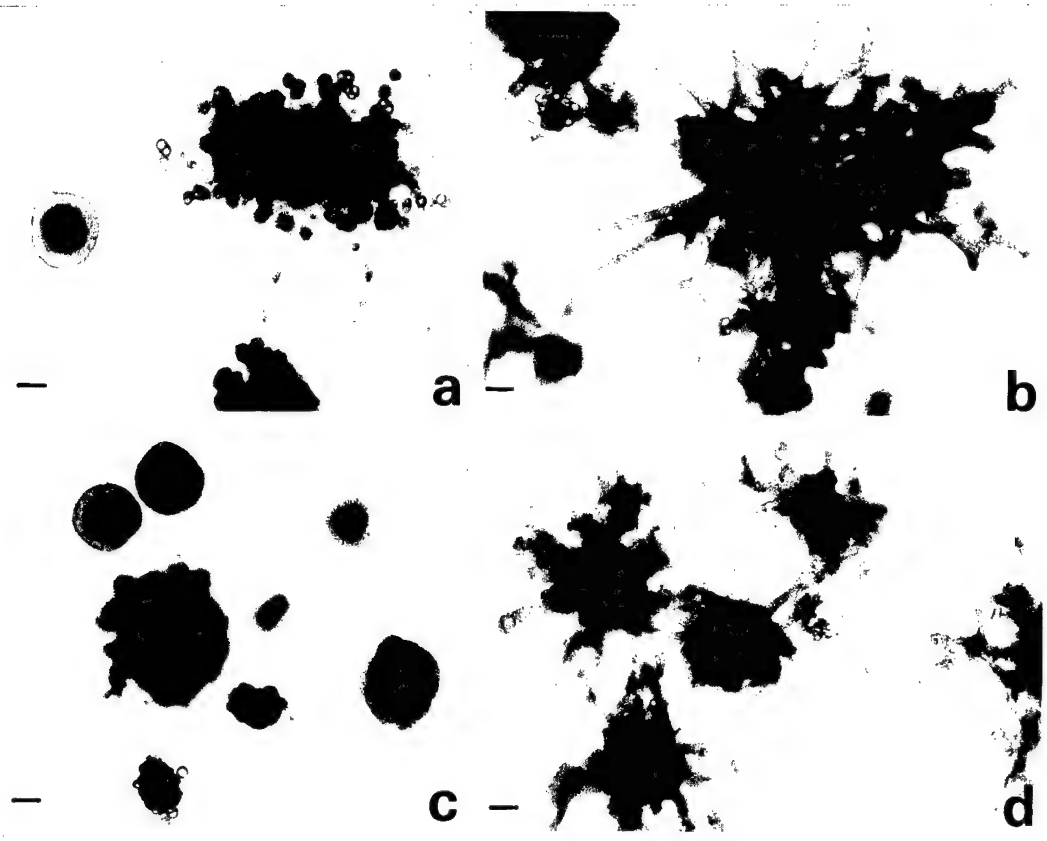


Figure 29

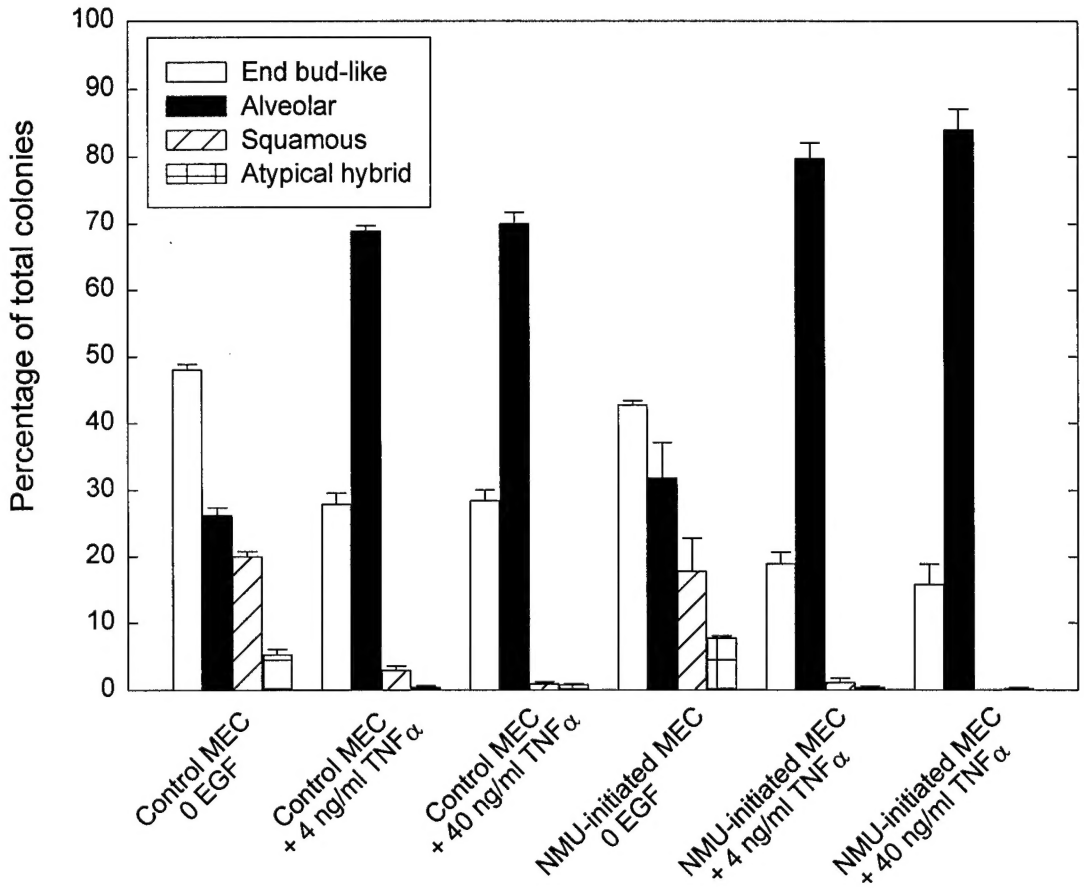

Percentage of total colonies
100
90
80
70
60
50
40
30
20
10
0
End bud-like
Alveolar
Squamous
Atypical hybrid
Control MEC 0 EGF
Control MEC + 4 ng/ml TNFα
Control MEC + 40 ng/ml TNFα
NMU-initiated MEC 0 EGF
NMU-initiated MEC + 4 ng/ml TNFα
NMU-initiated MEC + 40 ng/ml TNFα

Figure 30

Figure 31

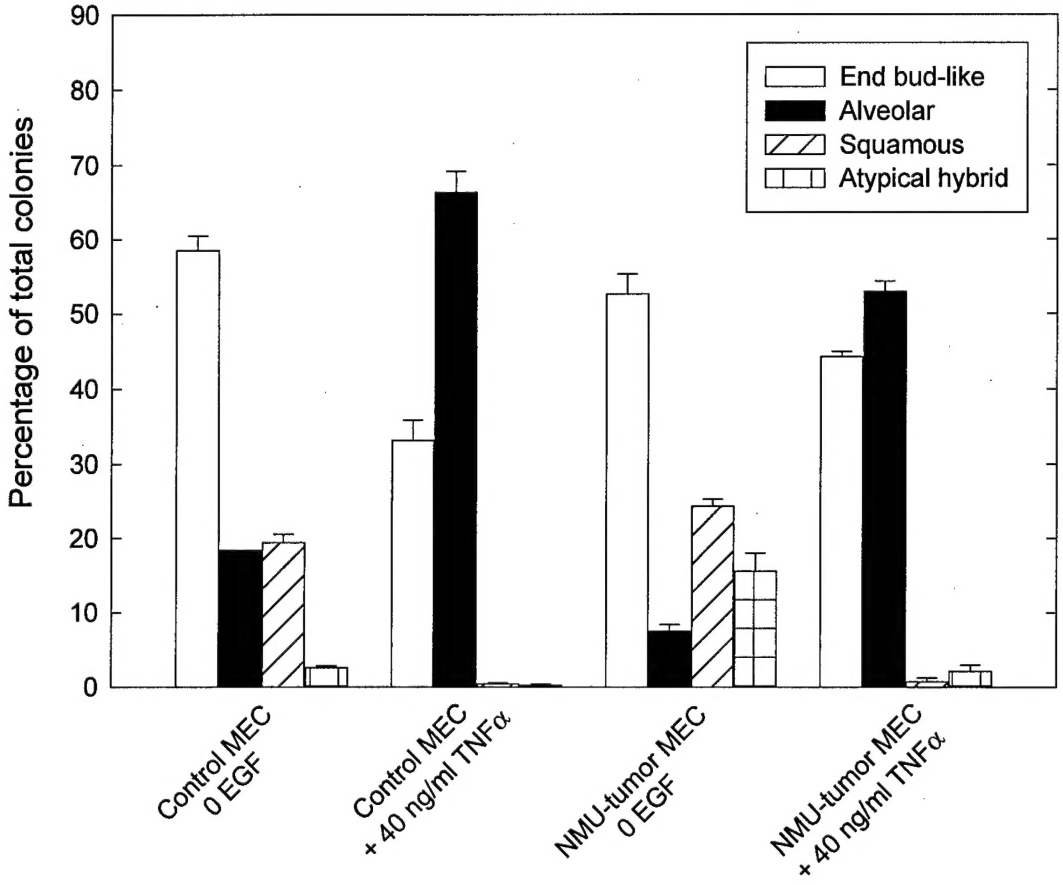

Percentage of total colonies
90
80
70
60
50
40
30
20
10
0
End bud-like
Alveolar
Squamous
Atypical hybrid
Control MEC
0 EGF
Control MEC
+ 40 ng/ml TNFα
NMU-tumor MEC
0 EGF
NMU-tumor MEC
+ 40 ng/ml TNFα

Figure 32

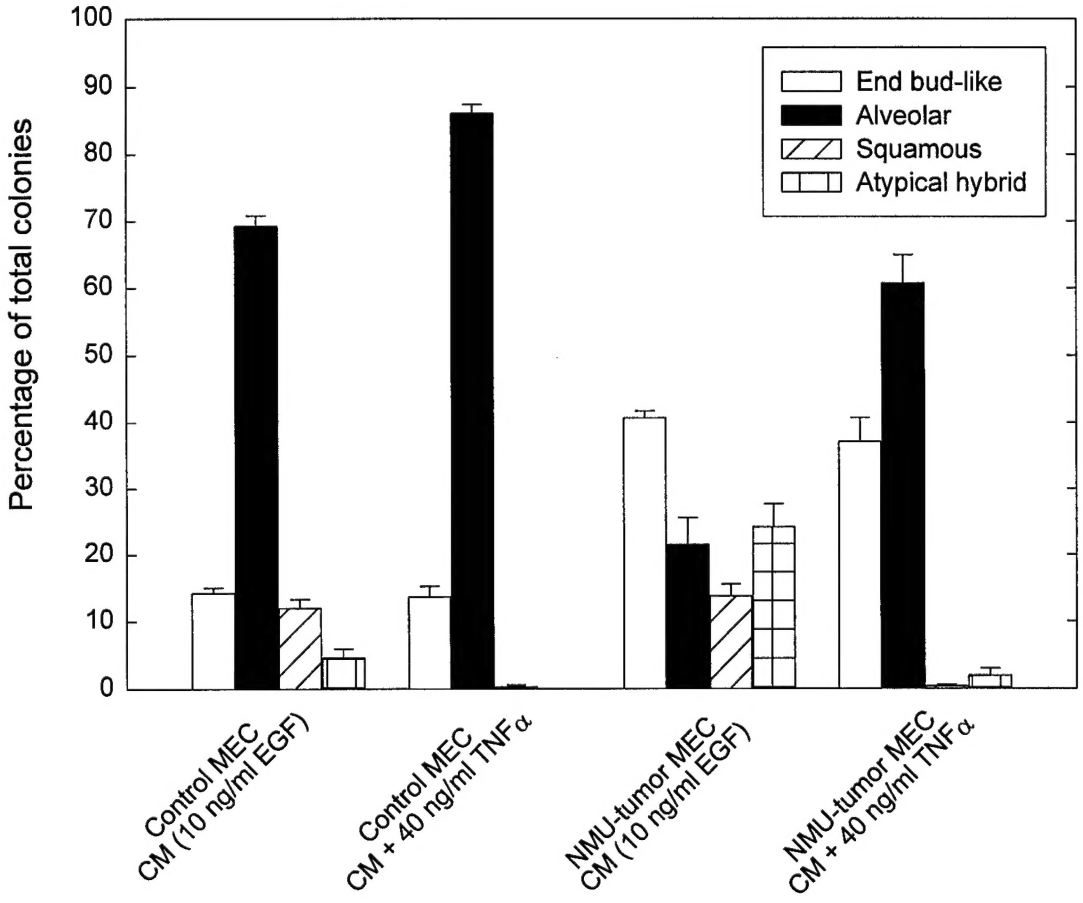

Percentage of total colonies
100
90
80
70
60
50
40
30
20
10
0
End bud-like
Alveolar
Squamous
Atypical hybrid
Control MEC
CM (10 ng/ml EGF)
Control MEC
CM + 40 ng/ml TNFα
NMU-tumor MEC
CM (10 ng/ml EGF)
NMU-tumor MEC
CM + 40 ng/ml TNFα

Figure 33